Role of MicroRNA-29 and ADAM12 in the Regulation of REST Dependent Signaling Pathways in Uterine Fibroids

By

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Abstract

Uterine fibroids, also known as leiomyomas, are benign smooth muscle cell (SMC) tumors of the myometrium and are the most frequent reason for a hysterectomy. Although benign, these tumors pose a significant burden on patients with symptoms of abdominal pain, pressure, uterine bleeding, and infertility; creating a great liability for the US economy with an annual estimated cost of up to $34 billion. Currently there are no long-term treatments for fibroids that will leave fertility intact, mainly because the mechanism of pathogenesis of these tumors is largely unknown. One of the key characteristics of uterine fibroids is the excessive deposition and reorganization of extracellular matrix (ECM). Altered ECM, which amplifies growth factor signaling and disrupts mechanosensing, has been proposed to promote fibroid tumor growth. Analysis of available gene expression datasets from GEO database indicates that *ADAM12* expression is dramatically upregulated in fibroids. As a member of the A-Disintegrin And Metalloprotease family of matrix modifying enzymes, ADAM12 is known to cleave ECM proteins, activate epidermal growth factor (EGF) and Insulin-like growth factor (IGF) signaling and promote tumorigenesis. Our lab has recently shown the expression of RE1 suppressing transcription factor /neuron-restrictive silencing factor (REST/NRSF), a known tumor suppressor, to be lost in fibroids. Upon further analysis, we found silencing *REST* in primary myometrial SMCs led to an increase in a number of downstream target genes, a profile similar to what is seen in fibroid tumor samples. Furthermore, Ingenuity® pathway analyses of gene expression datasets for fibroids indicate that the loss of REST and the increase in ADAM12 expression in leiomyomas could be linked through microRNA-29 (miR-29). In addition, in cultured mammary tumor cell lines, miR-29 is known to directly regulate ADAM12 expression. Compared to normal myometrium, uterine fibroids express significantly lower levels of miR-29.

In its promoter, miR-29 contains an RE1 element, a putative binding site for REST. In order to see how the loss of REST in fibroids could affect miR-29, we silenced REST in primary myometrial SMCs and saw a significant decrease in miR-29 expression. In addition, Western blot analysis showed an increase in ADAM12 expression and EGF receptor (EGFR) phosphorylation when REST was knocked-down in primary myometrial cells. We further investigated the link between miR-29 and ADAM12 expression by treating primary myometrial and leiomyoma cells with miR-29 inhibitors and mimics, respectively. We found that inhibiting miR-29 in myometrial cells results in an increase in the expression of ADAM12. Conversely, the opposite effects were seen when fibroid cells were treated with miR-29 mimics. Furthermore, overexpression of ADAM12 in primary myometrial SMCs was found to induce activation of multiple tumorigenic signaling pathways including EGFR, Mitogen-activated protein kinase (MAPK), the phosphatidylinositol 3-kinase (PI3K), protein kinase B (PKB/Akt), and mammalian target of rapamycin (mTOR) and Notch pathways. In conclusion, we report a novel druggable pathway that links the loss of REST, down regulation of miR-29, increased ADAM12 expression, and the activation of multiple tumorigenic pathways in uterine fibroids.

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Dedicated to my parents

Hadi and Debbie Farahbakhsh.

Thank you for always believing in me and helping me reach my dreams.

Table of Contents

Chapter I: General Introduction of Uterine Fibroids

I. Overview

Uterine fibroids, also known as leiomyomas, are the most common benign tumor of the female reproductive tract. It is estimated that most women, 70% of Caucasians and 80% of black women, will have a fibroid before reaching menopause and one third of these women will require medical treatment [1, 2]. In addition to being a major public health problem, uterine fibroids put a great burden on the US economy with an estimated annual cost of $34 billion [3]. Despite their high incidence, little is known about their etiology. However, in recent years research in the area of fibroids has increased, leading to important breakthroughs in their pathogenesis. Over the years there have been predisposing factors recognized for uterine fibroids, familiarity with these risk factors can aid in better understanding the etiology of the tumors.

II. Clinical Presentation, diagnosis and Management

1. Classification

The International Federation of Gynecology and Obstetrics (FIGO) has developed a classification system of fibroids which is based on the location of the tumors and their effect on abnormal uterine bleeding. The scoring system includes Types 0-8, where type 0 is a submucosal pedunculated fibroid (tumor growth on a stalk), types 1-2 are submucosal (projecting within the uterine cavity), types 3-4 are intramural (located within the myometrium), types 5-6 are subserosal (projecting outside the uterus), type 7 is subserosal pedunculated and type 8 is all other sites such as cervical. The types 1-6 are determined based on the percentage of the fibroid located in the intramural position [4]. Having a commonly used classification system

will help all clinicians and researchers to have a better understanding of the characteristics of each fibroid and the symptoms they cause.

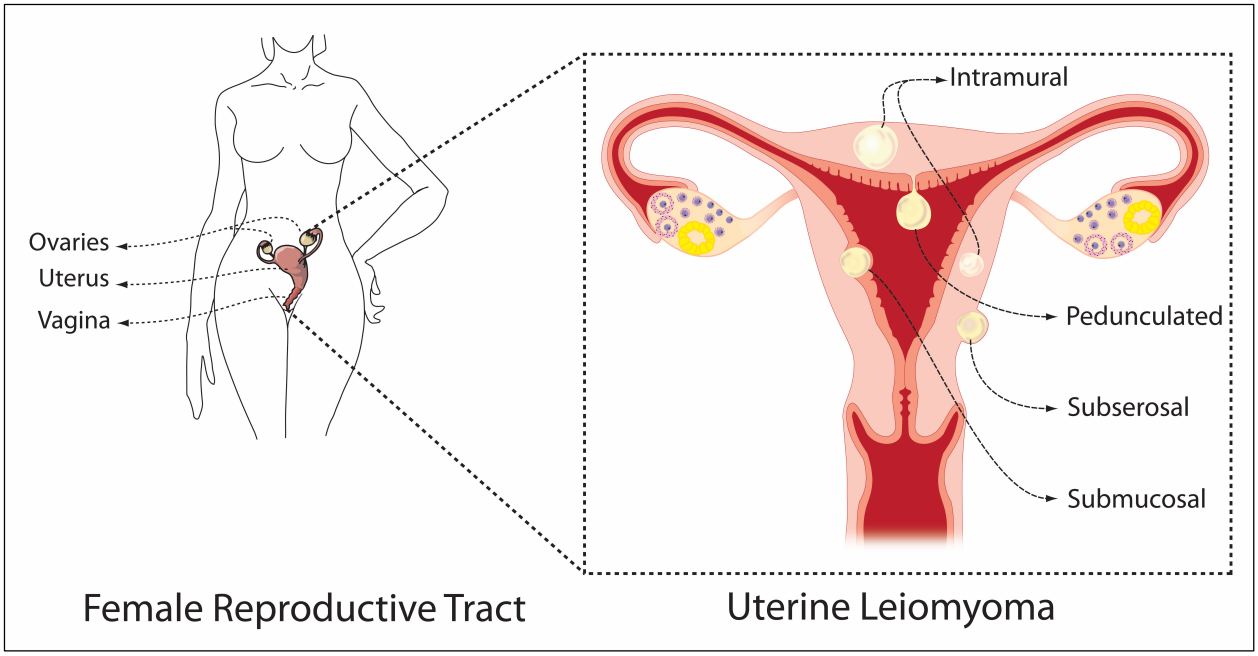

Figure 1: Representing the different locations of fibroids in the uterus for their classification; Submucosal (fibroids projecting within the uterine cavity), Intramural (fibroids located within the myometrium), Subserosal (fibroids projecting outside the uterus) and pedunculated (fibroids growing on a stalk).

2. Symptoms

Fibroids can cause severe morbidity for women. Women can suffer heavy menstrual bleeding which can cause iron deficiency. Larger fibroids can cause bulk symptoms, including urinary retention, frequent urination and nocturia. Additional bulk symptoms include gastrointestinal problems, pain and abdominal distention. In addition, fibroids are thought to be associated with infertility [5]. Furthermore, evidence suggests a link between uterine fibroids and spontaneous abortion, preterm labor, malpresentation and postpartum hemorrhage in pregnancy [6]. In many cases women with fibroids can also be asymptomatic. In addition to clinical symptoms it is important to consider the burden due to indirect costs of uterine fibroids on the patients. Women with fibroids can lose household income due to loss of work and disability when suffering from symptoms of their tumors. Other costs can include over the counter medications, alternative therapies, hygienic products and adult diapers which are needed for heavy bleeding and incontinence. The estimated annual cost for heavy menstrual bleeding due to all causes is $1,700 per woman. Furthermore, patients may suffer from infertility and will be in need of further infertility treatments which will add to the cost and suffering of the patients [7].

3. Diagnosis

3.1. Imaging

The diversity among uterine fibroids makes them difficult to diagnose. The tumors vary in size, location and numbers per patient. Therefore, the symptoms between patients will also vary. In the case of uterine fibroids, a clinical pelvic examination finding a firm and multilobular uterus or a firm palpable mass bulging from the uterus strongly suggests the presence of fibroids. However, for the clinical diagnosis, imaging is most commonly used. Pelvic ultrasonography is the gold standard for detection of uterine fibroids. This technique is favored due to its

availability, low cost and ease of use [8]. Transvaginal sonography for the diagnosis of submucosal fibroids had a sensitivity and specificity of 90% and 98% respectively. The addition of sonohysterography (injection of saline into the uterine cavity) to this technique increases the sensitivity and specificity of fibroid detection to 100% [9]. X-ray examination is another imaging source available for uterine fibroid diagnosis. However, the sensitivity and specificity of this method drops down to 50% and 20% respectively [4]. Magnetic resonance imaging (MRI) is another useful tool for fibroid diagnosis with a sensitivity of 99% and a specificity of 86%. Due to the higher cost of MRI, it is recommended to be used when exact mapping of the fibroid is beneficial, for example before surgery, and also in cases of patients with larger uteri and multiple tumors [10].

3.2. Histology

Uterine fibroids are distinct from the adjacent normal myometrium. They present as an encapsulated mass with excess collagen deposition and are grossly firm and pale in color. Microscopically, they have spindle shaped cells with eosinophilic cytoplasm arranged in a whorled pattern with a higher mitotic rate than the adjacent normal myometrium. Uterine fibroids can also present as ulcerated, calcified, hemorrhagic and necrotic tumors based on their size and location. Many of these characteristics are seen in fibroids of pregnant women or patients taking progestin treatment [11].

4. Clinical Management Strategies

Although most of uterine fibroids are asymptomatic, 20-50% of them will cause clinical symptoms and require treatment. First line medical therapy for symptomatic fibroids include nonsteroidal anti-inflammatory drugs (NSAIDs) for reducing pain and menstrual bleeding and iron supplementation for management of anemia [12, 13]. When deciding on a treatment option,

it is important to individualize it towards each patient. The selection of treatment should be based on the symptoms, size and location of the fibroids, age and desire of patient to retain fertility, availability and expertise of the physician [8]. Additional therapeutic options for fibroids can be categorized in two groups: uterine sparing and non-sparing. The uterine non-sparing option includes hysterectomy and the uterine sparing treatments can be further sub-divided into medical and procedural/surgical branches.

4.1. Uterine - Sparing Medical Treatments

a. Progesterone Receptor Modulators

Uterine fibroids are known to be hormone-dependent tumors [14]. Progesterone receptor modulators (PRMs) competitively bind and inhibit progesterone receptors. Two of the most highly studied PRMs for uterine fibroid treatment are ulipristal acetate (UA) and mifepristone. Randomized control studies of mifepristone versus other treatments or placebo found mifepristone to be beneficial in reducing heavy menstrual bleeding and improving quality of life, however, it did not reduce the size of fibroids or uterus [15]. Treatment with UA has recently been approved by the US Food and Drug Administration for short-term preoperative therapy [16]. A randomized, double-blind and placebo controlled clinical trial was carried out among 38 research centers in six countries called the PEARL I study group (PGL4001 (UA) Efficacy Assessment in Reduction of Symptoms Due to Uterine Leiomyomata). The study showed greater than 90% of women had controlled uterine bleeding and a 20% reduction in fibroid size when treated with UA for 13 weeks. In addition, induced benign changes in the endometrium due to UA treatment were resolved by 6 months after end of treatment [17]. Longer treatments with four rounds of 12 week periods were also conducted in PEARL III and IV studies where

they saw positive outcomes in uterine bleeding and fibroid volume reduction with low side effects [18].

b. Antifibrinolytic agent

Tranexamic acid is an oral antifibrinolytic agent which is commonly used as therapy for heavy menstrual bleeding [19]. In a clinical trial where 115 women received tranexamic acid, there was a significant reduction in uterine bleeding and an improvement in quality of life determined by increased leisure and physical activities and work inside and outside the home [20]. A systemic review looking at 10 studies found results supporting the tolerance and benefits of tranexamic acid for treatment of heavy menstrual bleeding. Although, the mechanism of action of tranexamic acid as an antifibrinolytic agent brings concern for the risk of a thrombotic event, no association was found between the two in this systemic review [21].

c. Gonadotropin-Releasing Hormone Agonist

Gonadotropin-releasing hormone (GnRH) agonists are valuable in treating anemia due to excessive uterine bleeding and reducing the volume of fibroids and uterus. GnRH agonists are thought to act by down-regulating pituitary GnRH receptors, leading to a reduction in gonadotropin levels and downstream ovarian hormone production [22]. This hypogonadotropic hypogonadal state, also known as pseudomenopause, has many side effects. The hypogonadal state caused by GnRH agonist treatment can lead to hot flashes, vaginal dryness, decreased libido, fatigue, headache, depression and decreased bone mineral density [23]. Therefore, the use of GnRH agonists in patients with uterine fibroids are commonly restricted to a short period of time (3-4 months) for preoperative use to help reduce bleeding and volume of fibroids and limit difficulties during surgery [19]. A systemic review of randomized controlled trials, looking

at the benefits of GnRH agonist treatment preoperatively for uterine fibroids, found a short-term treatment led to a decrease in uterine and fibroid volumes. In addition, the treatment was able to correct preoperative anemia due to excess uterine bleeding and lower intraoperative blood loss as well. The use of GnRH agonists also made it possible for many women to undergo the less invasive, transvaginal procedure, instead of an abdominal route [24]. The transvaginal option has been shown to be more beneficial versus abdominal surgery due to a speedier recovery rate and lower risks of infection and fever [25].

d. Oral Contraceptives and Levonorgestrel-Releasing Intrauterine Device

Combination OCs (COCs) are commonly used for treatment of heavy menstrual bleeding. Certain benefits to use of COCs include their ease of administration, low side effects and affordability [26]. A recent meta-analysis looking at cohort and case-control studies for the use of OCs and risk of uterine fibroids, suggested the presence of fibroids should not be considered a contra-indication for OC use in patients [27]. Furthermore, a study comparing the effects of COCs and levonorgestrel-releasing intrauterine device (LNG-IUD), found both groups were able to reduce the amount of uterine bleeding in more than half the patients, however, the LNG-IUD group was more effective at reducing bleeding and also restored hemoglobin levels in patients [28]. In one observational study by Machado and colleagues the effectiveness of the LNG-IUD in avoiding the need for a hysterectomy was tested. This study included sixty perimenopausal women with uterine fibroids and excessive uterine bleeding with previous referral for a hysterectomy. From these women, 39 patients opted to use an LNG-IUD and the remaining 21 patients elected for a hysterectomy. After 24 months of follow-up, Machado et al. found 89.5 percent of the LNG-IUD patients avoided a hysterectomy and reported a greater satisfaction with their treatment compared to the hysterectomy group [29]. The LNG-IUD has been shown to be

effective in reducing heavy menstrual bladding in women with uterine fibroids, however, the LNG-IUD has been reported to be associated with increased risk of expulsion [30] and their use is contraindicated in patients with fibroids distorting their uterine cavity [31]. When treating symptomatic uterine fibroids it is important to tailor the treatment for each individual patient and provide them with all available options; explaining their limitation, benefits and drawbacks.

4.2. Uterine Sparing - Surgical Procedures

a. Myomectomy

Myomectomy is the surgical or endoscopic removal of uterine fibroids. The endoscopic myomectomy procedure involves the placement of an endoscope through the cervix to remove fibroids from the endometrial cavity. The use of a camera also aids in this process for direct visualization of the tumors, which is then referred to as hysteroscopic myomectomy. This procedure is favored due to its quick recovery rate and preservation of fertility. During laparoscopic myomectomy and hysterectomy an electric surgical device, known as a power morcellator, is commonly used to break down large pieces of fibroid tumors for easy removal [32]. It has recently been a concern that the use of a power morcellator in the presence of an unsuspected sarcoma could lead to the dissemination of cancer cells within the intraperitoneal cavity. In response, in 2014 the FDA released a statement warning against the use of a power morcellator for the removal of uterine fibroids in peri- and postmenopausal women or in premenopausal women who are a candidate for en bloc tissue removal [33]. In symptomatic women who have intramural or subserosal fibroids and wish to preserve their fertility, surgical myomectomy is recommended [32]. In the case of abdominal myomectomy there is a frequent formation of postoperative adhesions and increased chance of a need for hysterectomy, all of

which may affect the fertility of the patient [34]. For symptomatic intracavity and submucosal fibroids, hysteroscopic myomectomy is preferred. This is an outpatient procedure and women can return to work after a few days and is thought to increase chances of future pregnancy [32, 35]. A systemic review by Bhave Chittawar et al. found laparoscopic myomectomy to have lower postoperative pain and fever and shorter hospital stay compared to open myomectomy surgery [36]. The recurrence rate of fibroids after myomectomy is estimated to be 15-30% after five years, this rate depends on the size and number of fibroids resected [5].

b. Uterine Artery Embolization

Uterine artery embolization (UAE) is a non-surgical uterine sparing procedure for treating uterine fibroids. In this procedure an angiographic catheter is placed in the uterine arteries and embolic particles are injected within, decreasing the blood flow in the arteries. This decreased blood flow will cause ischemic injury leading to fibroid necrosis and shrinkage, but allowing for the normal myometrium to recover [19]. Compared to hysterectomy and myomectomy, UAE has a shorter hospital stay, decreased time to begin normal activities and a lower chance of needing a blood transfusion [5]. Currently the data on the effect of this procedure on future fertility are limited, therefore, UAE is not recommended for women desiring to become pregnant in the future. Although UAE is a minimally invasive procedure with short hospitalization stay compared to surgery, it is associated with higher rates of minor complications and an increased risk of need for surgery in 2-5 years after the initial procedure [37]. Complications from UAE can include ischemic pain in the first 12 hours after the procedure, ejection of infarcted fibroid pieces from the vagina and very rarely a case of intrauterine infection [19]. It is important to consult with each patient about the benefits and risks of the procedure and inform them of

possible need for future interventions and impact on fertility if they choose UAE for their treatment.

c. Endometrial Ablation

One method for treatment of heavy menstrual bleeding (HMB), is to use endometrial ablation to destroy the uterine lining. Compared to hysterectomy, endometrial ablation has been shown to be less invasive, have shorter hospital stays, a faster recovery rate, to be associated with less pain and fewer complications. The second generation devices used for endometrial ablation allow for outpatient procedures and include hydrothermablation, bipolar radiofrequency, endometrial cryotherapy, thermal balloon or microwave energy [38]. However, this method of treatment should only be considered for women who do not desire to have children in the future. In a study in 1993, 51 women underwent hysterescopic resection with or without endometrial ablation for treatment of HMB due to fibroids. It was reported that 2/3 of the women who had undergone endometrial ablation were amenorrheic after the procedure [39]. In a cohort study including 114,910 women who underwent endometrial ablation for treatment of HMB, the risk for further intervention was assessed by Bansi-Matharu and colleagues. They found 1 in 6 women required further surgery within 5 years, and this risk was decreased whith increasing age at time of initial treatment [40]. For the correct patient, endometrial ablation can be a great therapeutic option for HMB treatment. Thermal ablation has been shown to be 80-90% effective in reducing blood flow and 40-50% of these women become amenorrheic. In addition, with low reintervention rates the patient satisfaction is about 90%. It is important for physicians to discuss the risks and benefits of this procedure to their patients and also to see if their patients are a good fit based on their symptoms, presence of endometrial hyperplasia or malignancies, classification of fibroids and desire for future fertility [41].

d. Image-Guided High-Intensity Focused Ultrasound

Magnetic resonance-guided high-intensity focused ultrasound (MRgFUS) is a non-invasive image-guided procedure which uses high-intensity ultrasound waves to produce heat and destroy uterine fibroids. Ultrasound-guided high-intensity focused ultrasound is another type of Image-guided technique which functions similarly to MRgFUS. There are advantages and disadvantages to both methods. The use of ultrasound is more affordable, compact, allows for real-time imaging and is easily transportable. On the other hand, MRI imaging allows for multi-planar, large field and better quality images with available thermometry technique which leads to more accurate treatment and lower complications. In the United States MRgFUS is mainly used for treatment of fibroids [42]. MRgFUS procedure has a high exclusion rate, as it is recommended for intramural fibroids and cannot be used in cases where bowel loops or scars on the abdominal wall obstruct the pathway of the ultrasound beam [19]. One study looking at 783 women, found 60% of patients to be ineligible for MRgFUS due to a bowel interposition [43]. Women who had underwent a procedure with MRgFUS had a similar improvement in quality of life but fewer complications when compared to women with abdominal hysterectomy [44]. Quinn et al. did a cohort study with 280 women undergoing MRgFUS procedure for their fibroids, they found a 4% rate for minor complications which included urinary tract infection, urinary retention, buttock pain and vaginal bleeding. After a 5 year follow up of 162 of the women, they found a re-intervention rate of 59% [45]. Similar to UAE, little is known about the effect of MRgFUS on the fertility of patients and further studies are needed to understand the risks and benefits of this procedure to other available options.

e. Laparoscopic Ultrasound-Guided Radiofrequency Ablation

Laparoscopic ultrasound-guided radiofrequency ablation (RFA) is the application of heat to cause thermal elevation and tissue destruction. Heat can be applied by direct thermal conduction, electromagnetics or ultrasound. In 2012 FDA approved the use of the Acessa System for ablation of symptomatic uterine fibroids by direct ultrasound guidance [46]. In 2013 Chudnoff et al. performed a study to test the safety and efficacy of RFA for treatment of uterine fibroids. They included a cohort of 135 premenopausal women with symptomatic fibroids and used Acessa for their treatment. At 12 months follow up post RFA treatment, bleeding was reduced by 38% in these women. The baseline score for symptom severity was reduced from 61 to 26.6 and quality of life was increased from 37 to 79 at 12 months. In addition, the mean fibroid volume was reduced by 45% and 94% of women were satisfied with their treatment. There was only one case of a severe adverse event and one patient requiring reintervention [47]. A three year follow up of the previous study, including 104 participants, was carried out by Berman and colleagues. The patients reported a sustained improvement in symptom severity and quality of life at 36 months and intervention was only needed in 11% of the patients [48]. RFA appears to be an effective and promising tool for treating uterine fibroids with low side effects, quick recovery, improved life quality, symptom relief and high patient satisfaction. However, further studies are needed as there is currently lack of information on the effect of RFA on ability of patients to become pregnant after treatment.

4.3. Uterine Non-Sparing

a. Hysterectomy

The most common treatment for fibroids is hysterectomy, a major surgery which removes the uterus of women and eliminates their option of ever bearing children. Hysterectomy accounts for

76% of all procedures performed for fibroid treatment, and one half of all hysterectomies are due to fibroids [49]. Hysterectomy can be performed through different routes; transabdominal, transvaginal or laparoscopic. This is a treatment which cures uterine fibroids but should be used for women who do not wish to preserve their fertility. The method used for this surgery is important. Transvaginal and laparoscopic approaches have a lower loss of blood, pain and recovery time compared to the transabdominal approach, with transvaginal having the shortest recovery time. In addition, transabdominal surgery is correlated with higher surgery risks of infection, fever and febrile episodes [25]. In addition to surgical risks, hysterectomy can have other health problems for patients. In the United States, it is estimated 600,000 hysterectomies are performed each year and 64% of these surgeries occured with bilateral oophoterctomy in 2004 [50]. Bilateral oophorectomy leads to acute hypoestrogenism and hypoandrogenism resulting in premature menopause [51]. Work by Moorman et al. found premenopausal women undergoing hysterectomy with unilateral oophorectomy or those who retained both ovaries were at increased risk of early ovarian failure leading to early onset menopause. Early onset menopause has serious health concerns which include an increased risk for cardiovascular disease, osteoporosis and all-cause mortality [52]. Therefore, it is important to inform patients of risks associated with hysterectomy and provide them with alternative options which have lower side effects and conserve fertility for those patients who value it.

4.4. Resources

Below are resources listed which could be beneficial for the patient to become educated on uterine fibroids. In addition, resources where healthcare providers can find up-to-date information and guidelines to help provide the best care for the patients are included.

a. *Resources for patients:*

1. **Support and information:** Office on Women's Health and Fibroid Relief

   https://www.womenshealth.gov/a-z-topics/uterine-fibroids

   http://www.fibroidrelief.org/

2. **Symptoms:** National Institute of Child Health and Human Development

   https://www.nichd.nih.gov/health/topics/uterine/conditioninfo/pages/symptoms.aspx

3. **Treatment options:** WebMD

   http://www.webmd.com/women/uterine-fibroids/uterine-fibroids-treatment-overview#1

b. Resources for healthcare providers:

1. **Clinical Key:** Provides current information on medical and surgical content.
2. **Access Medicine:** Includes immediate access to up-to-date medical data.
3. **UpToDate:** Provides answers at point of care with access to evidence-based information.
4. **PubMed:** A valuable resource for health care providers to stay updated on research, clinical trials and case reports in science journal articles.

Most medications available for uterine fibroid treatment are restricted to use for short-term symptom relief and or preoperative purposes due to lack of effectiveness and or side-effects. There currently is no long-term and effective treatment option available for fibroids which will leave fertility intact. This is mainly due to our lack of knowledge on the pathogenesis of these

tumors. Therefore, there is a clear need to further our understanding of uterine fibroid formation for development of effective, affordable and widely available medications which can be used for long-term treatment and or cure of uterine fibroids.

III. Risk Factors

1. Race and Age

There is strong evidence supporting the increased risk of fibroids with increased age and being of black race. In 1989 a cohort of 95,061 premenopausal US nurses ages 25-42 years, lacking any previous diagnosis or history of uterine fibroid were included in a health study. Marshall et al. have successfully used information from these participants to understand the risk factors for uterine fibroids. The authors found a strong correlation between age and fibroids and black women were two to three times more likely to be diagnosed with fibroids compared to other races [53]. In addition, in a separate study black women were found to have developed fibroids at an earlier age compared to white women [1]. The National Hospital Discharge Survey 1988-1990 also found races other than white to be diagnosed with leiomyomas at higher rates and there were higher diagnoses with increased age of patients [54].

2. Age of Menarche

The early onset of menarche has been associated with an increased risk of fibroids. Ultrasound or hysterectomy confirmed diagnosis of fibroids in the Nurse Health Study II, showed a lower risk of fibroids for women who started menarche at 16 years or older compared to the average age of 12, while there was an increased risk for early onset of 10 years or younger. In addition, having regular cycles seems to correlate with a higher incidence of fibroid tumor formation [55]. The correlation between higher incidence of fibroids with regular menstrual cycles was also observed in a case-control study of 122 women undergoing hysterectomy in Japan for their fibroids [56]. Although not significant, others have also found a relationship between early onset of menarche and a greater risk of developing fibroids in premenopausal women [57, 58].

3. Parity

Parous women are at lower risk of having fibroids compared to nulliparous women [55, 57]. In Japan, 91 women undergoing hysterectomy for fibroids were analyzed. These women were more likely to be nulliparous compared to the control group and the increasing number of live births also decreased the risk of fibroids [59]. The Oxford Family Planning Association study including 535 women with pathologically confirmed fibroids was analyzed by Ross et al. where they found a decreased risk of fibroids with an increased number of term births. In addition, women who had at least five term-births had one-fourth the risk of developing fibroids compared to nulliparous women [60].

4. Menopause

The incidence of fibroids in pre- and postmenopausal women have been reported to be similar [57, 61]. However, Samadi et al. looked at 201 women in a case-control study where there was a self-report of fibroids with age matched controls and found a higher report of fibroids in the premenopausal group [58]. In addition to prevalence, a study looking at 100 hysterectomy specimen, sectioned at 2-mm intervals found the tumors in the postmenopausal women to be smaller and fewer in number in a single specimen [61].

5. Environmental Estrogen

Exposure to environmental estrogens appears to be associated with uterine fibroids. Baird et al looked at randomly selected women, including black and white participants, to examine the relationship between prenatal diethylstilbestrol (DES) exposure and uterine fibroid formation. They found the presence of fibroids in all five of the black women who had reported exposure to DES. In addition, 76% of white women who had reported neonatal DES exposure had a positive diagnosis for fibroids compared to 52% of women who had not reported any DES exposure. The authors also noted women who had been exposed to DES had larger fibroids as well [62]. In

another study, known as the NIEHS Sister Study, D′Aloisio and colleagues investigated the connection between early life exposure to estrogenic isoflavins in infant soy formula and uterine fibroid formation. Interestingly, looking at more than 19 thousand Caucasian women between the ages of 35 and 59, the investigators found women who had been fed soy formula as an infant had an increased risk of early fibroid diagnosis [63].

6. Oral Contraceptives

The effect of oral contraceptive (OC) use on fibroid formation seems to depend on the age when first used, the duration, amount and type of hormones used. In a study looking at pathologically confirmed fibroids from 535 women from the Oxford Family Planning Association, Ross et al. looked at the risk of fibroids in women using different types of contraception. They found the risk was lowered with continues use of OCs, and decreased 31% if taken for more than 10 years. When looking at the contents of the OC pills they found the higher the dose of progestogen the more protective effect it had, supporting the unopposed estrogen hypothesis [60]. In the Nurse Health Study II, Marshall et al. found the risk for fibroids was significantly increased for women who took OCs from the earlier ages of 13-16 compared to women who never took them [55]. In a study by Wise et al. 22,895 premenopausal women with intact uteri and no prior diagnosis of fibroids were followed for a period of 4 years and ultra-sound or hysterectomy were used for confirmation of diagnosis. They found the risk was decreased with current use of progestin-only injectable due to down regulation of estrogen receptors by progestin [64].

7. Obesity

There is an increased risk of fibroids in women with higher BMI and body weight. A study by Ross et al. found for every 10 kg of increased weight there was a 21% increased risk of developing fibroids [60]. In another study looking at 100 women undergoing hysterectomies for

their fibroids it was found those who had a BMI over 24 and a body fat percentage of 30 or greater were at higher risk of having fibroids. In addition, women with an upper body fat distribution with a larger waist to hip ratio were at greater risk [65].

8. Smoking

Smoking cigarettes seems to have a protective effect against fibroids. The nicotine in cigarettes reduces the conversion of androgens to estrogen through inhibition of aromatase. In addition, smoking reduces the bioavailability of estrogen by inducing the 2-hydroxylation pathway of estradiol metabolism [66, 67]. Parazinni et al. found that it was not the history of smoking but only current smokers who had a decreased risk. His study saw a 40% reduced risk in current smokers and work by Ross et al. also found a 2/3 less risk in women who smoked 20 cigarettes a day [57, 60].

IV. Etiology

Although currently the etiology of fibroids is unknown, several theories exist regarding important initiators of fibroids. One hypothesis states the mitogenic activity of progesterone and estrogen are responsible for tumor cell proliferation and somatic mutations and that the formation of fibroids is a complex interaction between these sex steroids and local growth factors [68]. Another theory is based off of Sampson's work in 1912, which states the abnormal bleeding in women is due to dysregulation of the vascular structure of the uterus. Stewart et al. hypothesize the growth factors which are involved in angiogenesis and vascular structures are responsible for the vascular abnormalities in fibroids. At time of vascular injury, factors such as basic fibroblast growth factors (bFGF) are increased and are important in cell proliferation. In fibroids, bFGF are also highly expressed and resemble a response to injury [69, 70]. An inherit abnormality in the myometrium has also been proposed as a possible etiology, as Richard et al. found the expression of estrogen receptors to be increased in the unaffected myometrium of uteri containing fibroids compared to non-fibroid containing uteri [71]. Another area of great research in etiology of fibroids includes genetics and epigenetics. Although, their role in the pathogenesis of fibroids should not be ignored, they do not seem to be the main culprit for formation of all fibroids. Cytogenetic analyses of uterine fibroids have revealed approximately 40% of tumors have non-random chromosomal abnormalities, which means the majority of them were chromosomally normal [72]. In addition, Marhsal et al. propose cryptogenic abnormalities occur as a second hit during the clonal expansion of tumors. In their study using short-term culturing of tumor cells, where both karyotypic normal and abnormal cells were present, the DNA showed a monoclonal pattern of X inactivation of the androgen receptor which was identical from the

fibroid of origin [73]. The proposition that cytogenetic changes may be secondary events has increased interest in genetic predispositions leading to uterine fibroid development.

1. Genetic Predisposition

Several studies support genetic predisposition for fibroids through the evaluation of twins, familial aggregation and association with hereditary syndromes. Looking at a random sample of 80 monozygotic and 80 dizygotic twins from the Finnish Twin Cohort, Luoto et al. found the reason for hospitalization due to fibroids was greater in monozygotic twins [74], supporting a genetic predisposition for fibroids. In further support, familial clustering of fibroids has been shown in multiple studies. In a study including ninety-seven families and 215 females, Vikhlyaeva et al. found fibroids to be twice as common in first degree relative females [75]. In addition, a study in Russia pointed to a greater risk of fibroids among proband's sisters and a study including Japanese middle aged women also showed a greater incidence of fibroids in women with a positive history in first-degree relatives [76, 77]. Another method for studying the genetic predisposition of fibroids is to look at hereditary disorders. Reed syndrome, also known as Multiple cutaneous and uterine leiomyomatosis, is an inherited autosomal dominate genetic condition. Females with this condition have larger and more numerous fibroids which develop earlier than the general population [78]. Women with Alport syndrome (ATS) an X-linked disorder with progressive nephropathy, are also commonly diagnosed with uterine fibroids [79]. The study of families with a genetic predisposition to renal cancers helped discover a germline mutation in fumarate hydratase, an enzyme of the Kreb's cycle, which results in a disorder known as hereditary leiomyomatosis and renal cell cancer (HLRCC). HLRCC is an autosomal dominant trait and patients with this disorder will suffer from renal cancer and leiomyomas of the skin and uterus [80]. The high incidence of fibroids in twin studies, first-degree relatives and

hereditary disorders in the mentioned studies provide support for the presence of genetic predisposition for fibroids.

2. Chromosomal Abnormalities and Genetic Mutations

2.1. Monoclonality

It is well accepted in the field that fibroids are monoclonal tumors. The initial studies to show the cellular origin of fibroids was done by Linder et al. where they looked at electrophoretic variants A and B of glucose-6-phosphate dehydrogenase (G6PD) in patient samples. As G6PD is X-linked, monoclonality can be determined by the exclusive presence of a single variant of the gene as the other variant will be silenced. The authors found the myometrium to have both variants A and B present in equal amounts while the tumors had either variant A or B of G6PD expressed [81]. Additional work by Townsend et al. confirmed these findings and further added that multiple fibroids from a single uterus could have either variant A or B of G6PD expressed [82]. These studies provide strong support for the monoclonality of fibroids and the distinctiveness of each fibroid in the same uterus.

2.2. Chromosomal Translocation and Deletion

Although uterine fibroids are not considered a genetic disease, cytogenetic studies have helped further understand the etiology of fibroids and have found approximately 40% of the tumors to have chromosomal abnormalities [83]. The most common chromosomal aberration, seen in approximately 20% of genetically abnormal fibroids, is a translocation between chromosomes 12 and 14, t(12;14) (q15;q23-24). The rearranged region of 12q has been shown to be important in other mesenchymal solid tumors. This region has been shown to include a highly conserved HMGIC gene which encodes a member of the heterogeneous high-mobility group (HMG) of

proteins [84]. HMGs are chromatin binding proteins, which can effect cell replication, transcription and repair [85]. Gattas et al. found HMGIC to be expressed only in fibroids containing the t(12;14). The expression of this protein may play a role in cell proliferation in growing tissue [86]. The next most common karyotype abnormality in fibroids, 17% of cases, is deletion of chromosome 7, del(7)(q22q32) [87] [72]. Recent work has shown changes in this region of chromosome 7 to target the cut-like homeobox 1 (CUX1) gene. This protein is responsible for modulation of expression of DNA damage response genes, and its abrogation can lead to increased DNA damage and strand breaks [88].

2.3. Genetic Mutation

Exome sequencing of a series of fibroid tumors revealed a high frequency mutation in the mediator complex subunit 12 (MED12) gene. This mutation has been reported in 50-85% of patient samples and is commonly detected in exons 1 and 2. MED12 is a transcriptional regulator that connects RNA polymerase II initiation complex to DNA regulatory sequences. Interestingly, it has been reported that MED12 mutations are usually present in the smaller size fibroid tumors and absent in the larger ones [89-91]. Recent work by Al-Hendy et al. has shown silencing MED12 in immortalized human uterine fibroid cell line (HuLM) using lentivirus carrying shRNA, leads to a significant loss of cell proliferation [92].

The chromosomal and genetic changes detected in at least half of the fibroid lesions, brings forth the question if these changes are primary to the formation of the tumors or are they secondary events? Further study is needed to understand the different pathways affected by these changes and how they contribute to the etiology of fibroids.

3. Epigenetics

3.1. DNA Methylation

The study of changes in phenotype due to altered gene activity and expression, without changing the DNA sequence, is known as epigenetics. The major classes of epigenetics mechanism are comprised of methylation and demethylation, microRNAs and histone modifications. DNA methylation commonly occurs in CpG islands near the promoter region. Hyper-methylation of this region leads to inhibition of gene expression while hypo-methylation activates gene expression [93]. The methylation of cytosine residues in the CpG islands occur by DNA methyltransferases by transferring methyl groups. The major DNA methyltransferases in mammals include DNMT1, DNMT3A and DNMT3B [94]. A study by Li et al. reported a state of DNA global hypo-methylation in uterine fibroids. Looking at 23 tumors and their adjacent myometrium, the authors detected either an increase or no change in DNMT1 expression in fibroids and a decrease in DNMT3A and 3B expression in more than 70% of the fibroids [95]. These data support a mechanism of epigenetic modulation as a part of uterine fibroid etiology.

3.2. MicroRNAs

MicroRNAs (miRNA) are another major class of epigenetic regulation. They can be classified as single-stranded, small non-coding RNAs of about 22 base pairs in length. These stable strands of RNA regulate gene expression through degradation of mRNA or inhibition of translation. miRNAs regulate their target genes by binding to their untranslated region (UTR) and their dysregulation has led to development of diseases and cancers [96-99]. The primary miRNA transcript (pri-miRNA) is formed by either RNA polymerase II or III transcription of the miRNA gene [100, 101]. The pri-miRNA transcript is then cleaved by Drosha in the nucleus to produce a precursor hairpin known as the pre-miRNA [102]. Once exported to the cytoplasm,

the Dicer complex cleaves the pre-miRNA into its mature length [103]. This mature form of miRNA is then loaded on to RNA-induced silencing complex (RISC), and together they bind to and regulate transcription and translation of target genes [104, 105]. Initial study by Wang et al. looking at miRNA expression in fibroids, examined expression patterns of 206 miRNAs in 41 patients. From this pool, 45 miRNAs were dysregulated compared to matched myometrium. They found the most highly dysregulated miRNAs to be the let-7 family, miR-21, miR-23b, miR-29b, and miR-197 [106]. In addition, a study by Marsh et al. looking at expression of 454 miRNAs in uterine fibroids, using microarray, found differential expression of miRNAs in fibroids and matched myometrium. Their findings included a cohort of 46 miRNA species, where 19 were over expressed and 27 were underexpressed in the tumors. Of these, only expression of miRNAs 21, 34a, 125b, 139, and 323 were confirmed by real-time polymerase chain reaction (PCR), where all were overexpressed except miR-139 which was underexpressed [107]. Recently Qiang et al. looked at the function of one of these underexpressed miRNAs, miR-29b, in more detail in fibroid pathogenesis. For their experiment they used female ovariectomized mice for subrenal xenograft model. They found the restoration of miR-29b in the xenograft tumors led to an inhibition of collagen synthesis and solid tumor maintenance. One of the major hallmarks of uterine fibroids is excess deposition of extracellular matrix (ECM), which is composed mainly of collagens I and III. Therefore, the authors were able to show the growth and maintenance of fibroids through production of ECM to depend on the down-regulation of miR-29b [108].

a. MicoRNA 29

The miR-29 family in humans has four members; has-miR-29a, has-miR-29b-1, has-miR-29b-2 and has-miR-29c. MiR-29a and 29b-1 are encoded by a gene on chr. 7q32.3 and miR-29c and

29b-2 are encoded by a gene on chr. 1q32.3 and each of these pairs are transcribed simultaneously. The mature forms of miR-29b-1 and 29b-2 have identical sequences and are known as miR-29b. All mature members of the miR-29 family share an identical sequence in their seed region, which is used for target recognition, and therefore share many of the same predicted targets as well [109]. Further work by Marsh et al. confirmed the dysregulation of the miR29 family in uterine fibroids. They showed their down-regulation to be important for increased collagen production in primary fibroid tumor cells and overexpression of miR-29 members led to a decrease in collagen expression in these cells [110]. Targeting miRNAs has become an area of interest for therapeutics. Manipulating a single miRNA could affect the regulation of many downstream target genes. There have been examples of both miRNA mimics and inhibitors entering clinical trials for therapy. A locked nucleic acid (LNA) targeting and inhibiting miR-122 has been used in clinical trials for the treatment of hepatitis C virus [111]. In addition, miR-34a mimics have entered trials while being packaged in lipid nanoparticles for easy delivery to cells [112]. Although targeting miRNAs is promising as a powerful tool for treatment of disease and cancers, it is important to strive for tissue specificity when designing therapeutics to minimize side-effects. Tools which could aid in this delivery method include viral vectors and direct injection into tissue of interest [113]. The continued research into the role of miRNAs in uterine fibroid pathogenesis and their potential as drug targets could lead to discovery of new and effective therapeutics for fibroids.

4. Hormones

The relationship between ovarian hormones and uterine fibroids has been extensively researched. Estrogen and Progesterone are cholesterol derived, steroid hormones that are essential for the development and function of the female reproductive system [114]. Numerous studies have

looked at the effect of these hormones and their receptors on fibroid growth. Strong evidence points to the estrogen dependency of fibroids. It is known that fibroids occur almost exclusively after puberty and they tend to shrink in size after menopause, in addition, treatment with gonadotropin releasing hormone (GnRH) agonists, which inhibit the production of ovarian hormones, leads to a decrease in growth and volume of the tumors [61, 115, 116]. Eker rats are used as an animal model for uterine fibroids. Due to a germline mutation in the *TSC2* gene these rats form spontaneous fibroid tumors [117]. Using fibroid tumor cell lines from Eker rats, Fuchs-Young et al. were able to show estrogen treatment led to increased proliferation and this increase was halted by the addition of estrogen antagonists. In addition, the antagonists led to an inhibition of estrogen-induced expression of progesterone receptors [118]. In order to exert its effect on target cells, estrogen binds to its receptors on the cell membrane or the nucleus. The nuclear receptors can be categorized as estrogen receptor α (ERα) and ERβ [119]. Studies have found the mRNA levels of ERα and ERβ to be increased in fibroids compared to normal myometrial tissue [120]. At protein levels, looking at different stages of the menstrual cycle, Kovacs et al. found ERα to be increased at all stages, while both forms of the nuclear ER were increased in the proliferative phase of the cycle. This relationship switched after menopause, with only an increase in ERβ and no difference in ERα expression levels in fibroids and normal myometrium [121]. With a major focus on estrogen, Ishikawa et al. changed gears to further understand the role of progesterone. Using a xenograft model, grafting human fibroid tissue under the renal capsule of immune deficient mice, they found the tumors grew in size when treated with estradiol and progesterone. The tumors grew by proliferation and an increase in cellular and extracellular volume. Interestingly, this effect was lost when progesterone was withdrawn. In addition, treatment of estradiol alone did not cause growth or maintenance of

tumor size. However, estradiol treatment did increase progesterone receptor expression. This study suggests progesterone to be required for the maintenance of volume and growth of fibroids while estradiol is needed to support progesterone function by increasing the expression of its receptors [122]. Progesterone also functions by binding to one of its two receptor isoforms, PRA and PRB, which are located on the nucleus [123]. Similar to ER, the concentration of PR has been shown to be increased in fibroids compared to normal myometrium at both mRNA and protein levels [124-126]. Looking at more detail at the difference between the forms of PR, Viville et al. found there was an increase in both PRA and PRB in fibroids, with a greater dominance of PRA [127]. The mechanism by which estrogen and progesterone affect the proliferation and growth of fibroids is still unknown. Research on these hormones has found them to influence many factors. Treating fibroid tumor cells with progesterone leads to an increase of epidermal growth factors (EGF). Interestingly, estrogen treatment decreases EGF levels but increases their receptors in tumor cells [128]. These data point to a collaborative relationship between progesterone and estrogen to increase fibroid cell proliferation through increase in EGF and EGFR. Matsuo et al. found progesterone to play a vital role in the expression of Bcl-2, an inhibitor of apoptosis, in fibroids. Bcl-2 is predominately present in fibroids in the secretory phase of the menstrual cycle, which is progesterone dominant. In addition, treatment of fibroid cells with progesterone led to an increase in Bcl-2 levels while estrogen treatment had an opposite effect [129]. Progesterone regulates other factors as well. It seems PRB negatively regulates the expression of insulin-like growth factor-I (IGF-I), while PRA has the reverse effect on IGF-I through blocking expression of PRB. Therefore, the ratio of these two PRs may determine their ultimate function in fibroids [130]. Other roles of estrogen in fibroids include increasing levels of platelet derived growth factors (PDGF) and lowering the

levels of myostatin and activin-A [131, 132]. In addition, estrogen has been reported to regulate proliferation of fibroid cells through increasing the activity of ATP-sensitive potassium channels [133].

5. Signaling Pathways

5.1. PI3K-Akt-mTOR Pathway

The complex cascade of signaling pathways is an important tool for transmitting a signal from binding of a single ligand to activating many downstream targets. Furthering our understanding of abnormalities in these pathways and their connections is important for potential therapeutic developments. Receptor tyrosine kinases (RTKs) are important components of signal transduction pathways and facilitate cell-cell communication. RTKs are type I plasma membrane receptors which are activated and dimerized by binding of their ligands. Once dimerized, the cytoplasmic domains of the RTKs are phosphorylated, leading to activation of downstream signaling pathways [134]. One such pathway is the phosphatidylinositol 3-kinase (PI3K), protein kinase B (PKB/Akt), and mammalian target of rapamycin (mTOR) pathway. In addition to being activated through RTK signaling, GPCRs and membrane-bound steroid receptors can also activate this signaling pathway [135]. Once activated, PI3K phosphorylates the plasma membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP_2) to phosphatidylinositol-3,4,5-trisphosphate (PIP_3) [136]. PIP_3 then recruits signaling proteins containing the pleckstrin-homology (PH) domain, including Akt. The signaling protein then activates downstream targets such as mTOR, Bcl-2 family proteins, glycogen synthase kinase 3 (GSK3), transcription factors and other molecules. The PI3K-Akt-mTOR pathway regulates important processes including cell proliferation, survival, cell cycle and apoptosis [137, 138]. This pathway is reported to be highly dysregulated in uterine fibroids and has been the subject of multiple studies. Using an

agonist of the progesterone receptor, Hoekstra and colleagues found an increase in fibroid cell proliferation with activation of Akt and GSK3. Furthermore, inhibition of Akt revoked the observed increase in cell proliferation. These results pointed to a role for progesterone in inducing fibroid cell proliferation through activation of Akt [139]. Using fibroid tumor samples from humans and the Eker rat model, Crabtree and colleagues found the mTOR pathway to be one of the most highly upregulated pathways in fibroids. They further analyzed this pathway as a therapeutic target in Eker rats by using an inhibitor of mTOR. Treatment for two weeks with rapamycin analogue, WAY-129327, led to a decrease in mTOR signaling and tumor cell proliferation. A longer treatment for a length of four months decreased the incidence, size and multiplicity of fibroids [140]; suggesting a role for the PI3K-Akt-mTOR pathway in the pathogenesis of uterine fibroids. Furthermore, Karra et al. looked at the activation status of the target genes of this pathway and found a significant increase in the phosphorylated (p) levels of GSK3 and cyclin D2 in fibroids [141]. More recent work by Varghese et al. showed a signaling protein, GPR10, upstream of the PI3K-Akt-mTOR pathway to be aberrantly expressed in human fibroid tissue samples. The activation of GPR10 led to induction of this pathway with an increase in fibroid cell proliferation in culture [142]. Based on these studies, modulating the PI3K-Akt-mTOR pathway could be a prospective target for fibroid treatment. Currently, an investigational drug, MK-2206, targeting Akt activity is in phase II trials for treatment of cancers [143, 144]. Sefton and colleagues have shown promising results in fibroids by treating tumor cells with MK-2206. The treatment caused reduced pAkt and induced cell death in cell culture and decreased growth of xenograft tumor cells [145]. Although there is strong evidence supporting a role for the PI3K-Akt-mTOR pathway in fibroid proliferation and pathogenesis, there are also groups whom have found conflicting results. Jeong and colleagues reported a

lower level of pAkt and PIP3 in fibroid tumors versus normal myometrium [146]. In addition, a more recent study by Makker et al. had similar findings where immunostaining showed a lower or absent pAkt and pmTOR in fibroids [147]. It is important to further study the regulators of this highly activated pathway in uterine fibroids to increase our understanding of its role in fibroid pathogenesis and possible therapeutic targets.

5.2. Notch Pathway

Notch is a cell-surface receptor which is involved in cell contact-dependent signaling. The Notch receptor family in mammals consists of four members, Notch 1-4. The ligands that bind to the Notch receptors are either Delta-like ligands (DLL-1, 3 or 4) or Jagged members (JAG-1, 2) [148]. Once bound by its ligand, the Notch receptor is cleaved by a member of the ADAM family, forming a truncated form of the transmembrane receptor [149]. This cleaved receptor is then further processed by the γ-secretase complex, releasing its intracellular domain (NICD). Once released, NICD travels to the nucleus where it can bind to nuclear effectors and regulate transcription [150]. Notch plays important roles in cell differentiation, proliferation, development and tissue homeostasis and its abnormal expression has been seen in many diseases [151]. Recently D'Angelo et al. have shown the regulation of breast cancer stem cells by Notch signaling [152]. Studies have also presented Notch signaling to contribute to non-small cell lung cancer pathogenesis through initiating lung cancer and enhancing epithelial-mesenchymal transition [153]. Notch-1 and its ligands DLL-1 and JAG-1, have been reported to be overexpressed in human glioma cells. Inhibition of Notch-1 and the ligands led to inhibition of proliferation and increased apoptosis in glioma cell lines [154]. Dysregulation of Notch signaling has also been seen in T-cell acute lymphoblastic leukemia, melanoma, lung adenocarcinoma and additional malignancies (Rizzo 2008). Although a role for Notch has not

been shown in fibroids, its connection to known dysregulated molecules makes this pathway a potential player in the pathogenesis of fibroid tumors. Additional studies are needed to further our understanding on the Notch pathway in uterine fibroids.

6. Growth Factors

Growth factors are proteins secreted by different cell types and function in autocrine and or paracrine fashion. They are known to bind to surface receptors on their target cells and activate downstream signaling pathways. Growth factors are important in controlling proliferation and growth of cells and their overexpression is evident in many cancers. A number of growth factors and their receptors have been identified in the myometrium and uterine fibroids. They include EGF, PDGF, HB-EGF, transforming growth factor ß (TGF- ß), vascular endothelial growth factor (VEGF) and IGF [2, 155].

6.1. HB-EGF

As part of the EGF family, HB-EGF is a 22-kD peptide, initially discovered to be secreted by macrophage cells and found to be a potent mitogen of smooth muscle cells (SMCs) [156]. HB-EGF is first synthesized as a 208 amino acid membrane-anchored precursor protein known as proHB-EGF. This precursor form contains a signal peptide, propeptide, mature HB-EGF, transmembrane and cytoplasmic domain. The proHB-EGF is then cleaved into the mature 73-87 amino acid soluble protein, HB-EGF. This mature form contains an EGF-like domain, which is a conserved cysteine rich domain present in all EGF family members. The EFG-like domain aids HB-EGF to bind its receptors and mediates its activity [157]. Several studies have reported an increase in HB-EGF levels and activity in different cancers [158]. Looking at human pancreatic cancer tissue, Kobrin et al. found an increase in HB-EGF expression in cancer compared to normal tissue. In adittion, treatment of pancreatic cancer cell lines with HB-EGF

enhanced their growth [159]. Cultured human bladder carcinoma cells were found to secrete growth factors including HB-EGF, which was involved in cell stimulation and growth in an autocrine manner [160]. Analyzing 100 cases of hepatocellular carcinoma (HCC) samples, Ito et al. observed a very low level of HB-EGF present in normal hepatic cells while the expression was much more prominent in early stages of HCC [161]. The expression of HB-EGF was also reported in melanoma, gastric tumors and breast cancer where it has been reported to have roles in their proliferation and growth [162-164]. In addition, HB-EGF is dysregulated in a number of fibrotic diseases [165-167]. With the presence of HB-EGF in a variety of cancers, diseases and with its mitogenic activity, it's important to further understand its role in their pathogenesis and to potentially use it as a therapeutic target for treatment. Initial studies on HB-EGF in the uterus was carried out by Zhang et al., where they looked at the effect of ovarian hormones on HB-EGF in the rat uterus. They found progesterone treatment led to an increase in HB-EGF expression in stromal cells and suspended its expression in luminal and glandular epithelial cells, while estrogen treatment strongly increased HB-EGF expression in epithelial cells [168]. Moving to human samples, Mangrulkar analyzed six matched patient fibroid samples and saw HB-EGF mRNA present in both tumors and myometrium, and their expression levels were lower in fibroids [70]. Further work by Wang et al. indicated treatment with HB-EGF led to proliferation of both myometrial and fibroid cells, however, myometrial cells responded to much lower concentrations of HB-EGF compared to tumor cells. In addition, HB-EGF is believed to prevent apoptosis in these cells through augmentation of Her-1 expression [169]. Currently the information on HB-EGF in fibroids is limited to low patient numbers and mRNA data. Further study is needed to look into the protein level of HB-EGF and their role in fibroid pathogenesis.

6.2. EGFR

HB-EGF functions by binding to its EGF receptor subtypes, HER-1 and HER-4 [170]. EGFR was discovered in 1978 by Carpenter et al. as the first RTK [171]. EGFR is a crucial part of cell biology; regulating proliferation, survival, tissue homeostasis, tumorigenesis and differentiation during development. In addition to HB-EGF, its ligands include EGF, transforming growth factor α, amphiregulin, betacellulin, epiregulin and epigen [172]. EGFR consists of multiple domains; an extracellular, transmembrane and a cytoplasmic domain with protein tyrosine kinase (PTK) activity [173]. Upon ligand binding, the receptor dimerizes and the PTK domain is autophosphorylated, providing a scaffold for binding of effector proteins [174]. The src homology 2 and phosphotyrosine binding (PTB) domain specifically bind to these phosphorylated sites on the receptor and activate downstream signaling pathways. These signaling cascades include the STAT, AKT, PI3K, phospholipase C gamma protein and the KRAS-BRAF-MEK-ERK pathways [175]. A number of studies have identified EGFR to be involved in pathogenesis of carcinomas by showing its overexpression in tumors. A role for EGFR has been seen in colon, mammary, ovarian, gastric, non-small-cell lung and other cancers [176]. The identification of EGFR in the myometrium and uterine fibroids was initially done by Hofmann et al. [177]. Shortly after, Fayed et al. used binding data from EGFR in myometrium and fibroids, and found a lower level of EGFR binding sites/cell in fibroids compared to myometrial cultures. In addition, there was an increase in DNA synthesis when both cell types were treated with EGF and insulin [178]. Their results were further confirmed by Tommola et al., where they observed decreased EGF binding and EGFR levels in fibroid tissue [179]. More recent work present conflicting results, Yu et al. used a phospho-receptor tyrosine kinase array and observed a 2 fold increased expression of EGFR in fibroid compared to myometrial tissue [180]. The differences in reported levels of EGFR expression in the mentioned studies may be

due to variation between samples, such as their location and or phase of the menstrual cycle. Additional work by Ren et al. represent a similar level of EGFR expression in fibroid and myometrial SMCs. Further analysis by laser scanning cytometry, showed an increase in DNA synthesis and polypoidization in fibroids after EGF treatment. Moreover, EGF treatment resulted in a transient state of EGFR and Akt activation in fibroid SMCs while the activation in myometrial cells was sustained. The authors propose this transient activation of EGFR could be due to lower affinity of EGF in fibroids and or higher activity of the tyrosine kinase, which may lead to rapid dephosphorylation and inactivation of the receptor [181].

7. Extracellular Matrix and Matrix MetalloProteinases

Uterine fibroids are known for their firm and nodular characteristic, which is due to excess accumulation of disorganized ECM. The ECM is composed of collagens and proteoglycans and normally functions to provide structural support and aids in intercellular communication [182]. In addition, ECM is involved in mechanostransduction, where by stretching and compressing the cells it surrounds, it can convert mechanical to chemical signals within these cells [183]. Initial work by Stewart et al. showed increased expression of collagens type I and III in uterine fibroids compared to matched myometrium [184]. Further studies found these collagen fibrils to be abundant, loosely packed and disoriented in fibroids [185]. Fibrosis is defined as a state of excess ECM production by cells which are resistant to apoptosis and have an increased proliferation rate, leading to highly crosslinked and disoriented collagen fibrils [183]. Based on this definition, uterine fibroids are fibrotic tumors, hence the name fibroids. Another common fibrotic lesion is keloids, also known as overgrowth of scar tissue. A study looking at molecular links between fibroids and keloids found both to have a decreased expression of dermatopontin, a collagen binding protein. In addition, ultrastructural analysis showed a strong resemblance in

collagen fibril orientation between the two fibrotic lesions [186]. The degradation and remodeling of ECM occurs by a special class of proteins known as the matrix metalloproteinases (MMPs). MMPs are also involved in cell growth, differentiation, apoptosis, migration , wound healing and inflammatory responses [187]. For their function, MMPs need to utilize Zn^{2+} in their active site and to be activated by cleavage of their propeptide [188]. Looking at MMP expression in the myometrium and fibroids, Wolańska et al. found MMPs-1, 2, 3 and 9 to be present in both groups. However, they saw much higher levels of MMP-2 in fibroids [189]. Additional work by Palmer et al. found an increase in MMP-11 in fibroids, while levels of MMP-1 and 3 were unchanged [190]. More recent work by Zheng et al. found MMP-9 to also be increased in fibroids [191]. MMPs can be regulated by their inhibitors, tissue inhibitors of matrix metalloproteinases (TIMPs) [192]. Having regulatory points for its activation and inhibition provides an equilibrium for the function of MMPs in different tissues. In addition to MMPs, TIMPs also target and inhibit the members of the disintegrin and metalloproteinase (ADAM) family of enzymes. ADAMs are cell-surface glycoproteins with proteolytic and cell adhesion activity. The multidomain structures of these proteins consist of a signal peptide, pro-domain, metalloproteinase domain, conserved disintegrin domain, cysteine rich domain, EGF-like domain, transmembrane region and the cytoplasmic tail [193].

8. ADAM12

ADAM12, a member of the ADAMs family, is present in two forms; a short secreted form (ADAM12-S) and a long transmembrane form (ADAM12-L). These two forms are products of alternate splicing of a gene on chromosome 10q26 [194]. ADAM12 is known to have a role in a variety of tissues. In the bone and cartilage, ADAM12 has been shown to regulate chondrocyte proliferation and promote bone elongation through proteolytic activity [195]. The formation of

multi-nucleated myoblasts is required for the development of skeletal muscle and Yagami-Hiromasa et al. showed ADAM12 aids in muscle formation through myoblast fusion [196]. In the liver and uterus, ADAM12 has been seen to have roles in ECM remodeling and decidualization [197, 198]. In addition to normal tissues, ADAM12 is involved in tumor development and other severe diseases. ADAM12 levels are dysregulated in breast cancer, and its urinary output directly correlates with cancer progression, therefore making it a useful prognostic marker [199]. In lung adenocarcinoma, ADAM12 levels have been shown to be an independent prognostic factor and correlated with tumor stage and post-surgery recurrence [200]. In addition to cancers, ADAM12 has a role in fibrotic diseases. ADAM12 has been indicated to promote liver fibrogenesis and also induce hypertension and cardiac hypertrophy [201, 202]. An increase in ADAM12 expression was observed in the airway epithelium of allergic patients after exposure to allergens and in bronchial epithelial tissue in response to proinflammatory stimulation, showing the dysregulation of this proteolytic enzyme in asthma [203]. Microarray and real-time polymerase chain reaction (qPCR) data present an increased expression of ADAM12 mRNA in uterine fibroid compared to normal myometrium [204]. With the involvement of ADAM12 in different cancers and diseases, there has been interest in finding ways to target and inhibit the activity of this enzyme for therapeutic purposes. ADAM12 has been reported to increase the availability of ligands of growth factor receptors through its catalytic activity. Shi et al. has shown ADAM12 to cleave the insulin-like growth factor-binding protein-3 (IGFBP-3) and increase bioavailability of the insulin-like growth factor (IGF) [205]. Looking at other members of the IGF binding protein family, Loechel et al. reported ADAM12 to also cleave IGFBP-5 [206]. One of the ligands and activators of EGFR is the heparin-binding epidermal growth factor (HB-EGF), which is cleaved from its membrane-bound form (proHB-

EGF), also known as "shedding", by ADAM12. Shedding of HB-EGF by ADAM12 makes it available to bind and activate its receptors [207]. Naturally the body controls and inhibits the activity of ADAM12 through TIMPs. Among the members of the TIMP family, TIMP-2 and 3 are the strongest inhibitors of ADAM12 [208]. Synthetic inhibitors have also been developed which inhibit the catalytic activity of ADAM12. KB-R7785 is one such inhibitor, which was shown by Asakura et al. to block ADAM12 activity and cardiac hypertrophy. This inhibitory function was due to KB-R7785 binding directly to ADAM12 and stopping it from shedding HB-EGF [207]. Using computational screening of a focused virtual library and activity assay, Oh et al. found four compounds, targeting Zn^{2+} binding site, to be potent inhibitors of ADAM12 [209] When designing inhibitor drugs it is important to have specificity to the enzyme of interest. A more recent study by Miller et al. tried to address this issue by designing a drug targeting the recombinant prodomain of ADAM12. They found their inhibitor to not cross-react with other ADAM members, and to specifically target ADAM12 activity and decrease shedding of HB-EGF [210]. Inhibiting ADAM12 with a drug has great potential as a therapeutic option for many patients. Further study is needed to understand the specific role of ADAM12 in disease processes and to find ways to target it specifically.

9. REST

The zinc finger transcription factor repressor element RE1-binding transcription factor (REST), also known as the neuron-restrictive silencer factor (NRSF), is a transcriptional regulator initially discovered by Chong et al. in 1995 [211]. REST inhibits the expression of neuronal genes by binding to the RE-1 binding site/ neuron-restrictive silencer element (RE-1/NRSE) on its target genes [212]. In a genome-wide analysis, Bruce et al. identified 1,892 potential targets of REST containing the RE-1 site in humans [213]. REST contains two repressor domains, RD1 on the N-

terminal domain and RD2 on the C-terminus. RD1 recruits corepressor Sin3A and histone deacetylase (HDAC) to the promoter of its target genes, while RD2 interacts with CoREST as a corepressor for REST [214, 215]. Initial work by Palm et al. looking at the rat brain found REST expression to decrease during neuronal development, but it was also expressed in the adult nervous system. The highest levels of REST mRNA were found in the hippocampus, midbrain and the pons/medulla [216]. More recent studies in humans, found contradicting information. Lu et al. measured REST levels in young adults (20-35 years) and old age (73-106 years) non-Alzheimer disease individuals. They discovered REST to be significantly increased in the ageing pre-frontal cortex at the mRNA and protein levels and it correlated with cognitive preservation and longevity. Interestingly, they found REST to repress genes involved in cell death and pathogenesis of Alzheimer's disease and to also induce the expression of genes involved in stress response. Indicating a protective role for REST in the adult nervous system [217]. As a regulator of gene expression, abnormalities in REST can lead to many problems. Studies have shown the aberrant expression of REST can result in opposite outcomes in the nervous and non-nervous systems. In human glioblastoma multiforme (GBM), REST acts as an oncogene where its expression is enriched and aids in self-renewal property of the GBM tumor cells [218]. In contrast, in colorectal cancer REST was identified as a tumor suppressor by an RNAi-based genetic screen, and cells lacking REST showed increased activity of the PI3K pathway [219]. A study by Varghese et al. identified G protein-coupled receptor 10 (GPR10) to be aberrantly expressed in fibroids and its activation to promote PI3K-Akt-mTOR pathway and cell proliferation. In addition, they report REST as the transcriptional repressor of GPR10 in the myometrium, and the loss of REST in fibroids leads to increased expression of GPR10 [142].

Objectives of Our Research

Uterine Fibroids are the most common tumors of the female reproductive tract and occur in up to 70% of reproductive age women [1]. These tumors cause great health and economic burden for the patients. Women with fibroids suffer from bleeding, incontinence, pain and infertility and can have up to $1700 in indirect costs annually due to symptoms [7, 32]. In addition, uterine fibroids are estimated to cost the US economy $34 billion on an annual basis [3]. Although uterine fibroids are very prevalent, and cause great burdens for patients and the economy, there are currently very limited options available for their treatment. The lack of understanding of mechanisms involved in fibroid pathogenesis is the main reason for this treatment limitation. By increasing our knowledge of uterine fibroid etiology, we could find potential drug targets for development of effective, affordable and long term treatment options which would leave fertility intact for the large number of patients suffering from uterine fibroids. In an attempt to uncover molecular culprits involved in fibroid formation, our lab recently discovered a tumor suppressor, RE1 suppressing transcription factor (REST), to be lost in uterine fibroids. As a transcriptional regulator, the loss of REST in fibroids can lead to aberrant expression of many downstream genes. Therefore, it is crucial to further our understanding on the significance of loss of REST on its target genes and signaling pathways on uterine fibroid pathogenesis. In this dissertation, we demonstrate A-Disintegrin and Metalloprotease family of matrix modifying enzymes, ADAM12, to be highly dysregulated in fibroid tumors. Our work suggests a role for ADAM12 in fibroid pathogenesis through activation of important tumorigenic signaling pathways. In addition, we report a novel pathway in uterine fibroids linking the dysregulation of ADAM12 and the loss of REST through the aberrant expression of microRNA-29 (miR-29). We show for the first time, a role for REST as a direct inducer of miR-29 and indirect suppressor of ADAM12 expression in

fibroids. Our findings provide new insight into important players and signaling pathways in uterine fibroid pathogenesis, and potential new targets for drug therapy development.

Chapter II: Regulation of REST Target Genes in Uterine Fibroids

I. Abstract

Uterine fibroids are the most common tumor of the female reproductive tract and the number one reason for hysterectomy. Although common, their pathogenesis is currently unknown. Our lab has shown the expression of RE1 suppressing transcription factor (REST), a known tumor suppressor, is lost in fibroids. Analysis of gene expression datasets indicated that many of the most abnormally expressed genes in uterine fibroids are known targets of REST. Using matched human fibroid and myometrial specimen, we analyzed the expression level of REST target genes, *SCG2, GRIA2, NEFH, SALL1, GRIN2A, STMN2, DCX and CBLN1* and found them to be overexpressed in the tumors. Furthermore, silencing REST in primary myometrial cells led to an increase in the expression of REST target genes, suggesting that the loss of REST leads to the aberrant expression of many of these genes in fibroids.

II. Introduction

Uterine fibroids are monoclonal and common tumors in women of reproductive age. They arise from smooth muscle cells (SMCs) of the myometrium and are characterized by excess deposition of extracellular matrix (ECM) consisting of collagens, proteoglycans and fibronectins. Fibroids can present as a single or multiple tumors in a patient, each of them clonally distinct, and can vary in location and size. They are classified based on their closeness to the layers of the uterus; subserosal, intramural and submucosal [220]. Patients with fibroids can present clinically with heavy bleeding which can result in anemia and bulk symptoms such as abdominal pressure and incontinence and in some cases the tumors can cause infertility and miscarriages in patients [32]. A study by Baird et al. using ultrasonography to randomly screen premenopausal women between the ages of 35 to 49 found an estimated cumulative incidence for fibroids before the age of 50 to be 70% for Caucasian women and more than 80% for black women [1]. Being of black race and increasing age up to menopause are the major risk factors for fibroids. In addition, early menarche, the use of oral contraceptives and a high-body-mass index are associated with an increased risk while increasing parity and a diet rich in vegetables, fruits and low-fats are associated with a decreased risk of fibroids [32]. It is estimated uterine fibroids cost the US economy $34 billion annually [3]. It is important to also consider the burden of indirect costs on patients. In addition to loss of income from missing work because of symptoms, the average woman with fibroids spends $1,700 a year due to heavy menstrual bleeding. This cost can include sanitary products, over the counter medications and alternative therapies [7]. Although uterine fibroids are very common and cause significant morbidity for the women they effect, their treatment options are limited. This is mainly because the pathogenesis of these tumors is unknown. Currently the most common performed procedure for uterine fibroid treatment is

hysterectomy, although it has been reported to be the least favored option among patients. A study by Borah et al. found of 96,852 procedures being done for fibroids, 76% of them included hysterectomies [49]. This procedure not only eliminates the ability of these women to ever bear children again, but is also associated with major health problems [51]. There is clearly a need to better understand the mechanism of uterine fibroid formation to help us provide patients with long-term and effective therapeutic options that will leave fertility intact.

In an attempt to better understand the pathogenesis of fibroids, our lab recently found the repressor element RE1-binding transcription factor (REST), also known as the neuron-restrictive silencer factor (NRSF), a known tumor suppressor to be lost in fibroids. Varghese et al. report on the role of REST as a transcriptional repressor of G protein-coupled receptor 10 (GPR10), which they found to be aberrantly expressed in fibroids. In addition, GPR10 activation was found to lead to downstream activation of the phosphatidylinositol 3-kinase (PI3K), protein kinase B (PKB/Akt), and mammalian target of rapamycin (mTOR) pathway. This pathway has been found to be highly activated in fibroids and thought to be involved in its pathogenesis [142]. As a transcriptional suppressor, REST regulates expression of many genes. However, little is known about the role of REST in fibroids. In an effort to find signaling molecules down stream of REST which could be effected by its loss of expression, we looked at human fibroid and myometrial samples from dataset GSE13319. In our search we found many REST target genes to be highly dysregulated. We found the genes to be aberrantly expressed at mRNA and protein levels, when we looked at their expression in fibroid specimen. Furthermore, we were able to show the role of REST in the dysregulation of these genes by silencing REST in primary myometrial cells. Our results show for the first time the loss of REST in uterine fibroids is

leading to the over-expression of a number of genes which could have important roles in tumor pathogenesis (Fig. 2).

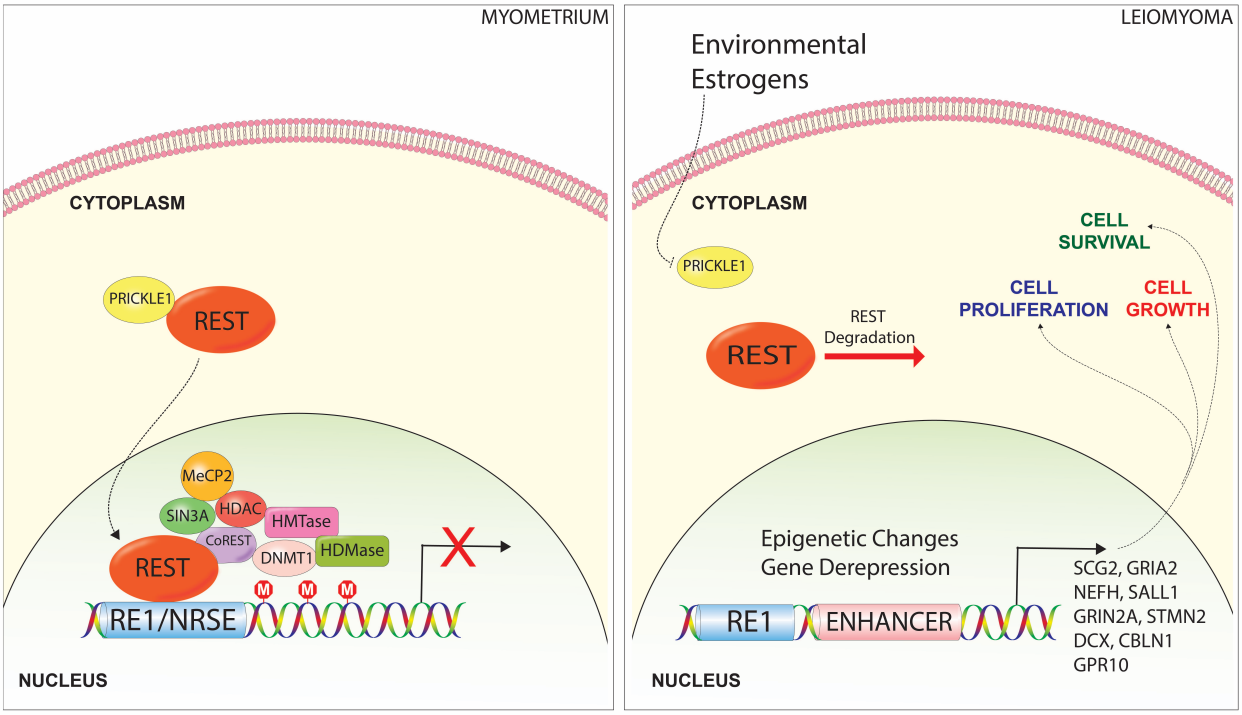

Figure 2: Working model depicting loss of REST through degradation in uterine fibroids (leiomyoma), leading to loss of inhibition and overexpression of downstream target genes.

III. Results

1. REST Target Genes Are Aberrantly Expressed in Uterine Fibroids

In an effort to further understand the effect of loss of REST in uterine fibroids, we looked at the expression profile of known REST target genes in the GEO database. In our search, using dataset GSE13319, we found many of the aberrantly expressed genes in human fibroids to be targets of REST (Fig. 3). In order to confirm these findings, we analyzed the expression of the genes in human fibroid and myometrial specimen in the lab. Using TaqMan RT-PCR we found mRNA levels of the REST target genes, sal-like protein 1 *(SALL1),* N-methyl D-aspartate 2A *(GRIN2A),* stathmin-like 2 *(STMN2),* glutamate receptor, ionotropic, AMPA 2 *(GRIA2)*, and Doublecortin *(DCX)* to be significantly upregulated in fibroid samples compared to paired normal adjacent myometrium. Additionally, neurofilament heavy polypeptide (*NEFH),* cerebellin 1 *(CBLN1)* and secretogranin II *(SCG2)* had an upward trend in expression in fibroid samples but the difference was not statistically significant; P= 0.08, P= 0.17 and P= 0.51 accordingly (Fig, 4A). After many failed attempts to specifically detect all of the REST target genes by Western blot using commercially available reagents, we were only successful at detecting GRIA2 and DCX. Western blot analysis showed GRIA2 and DCX protein levels to be highly expressed in fibroid samples compared to normal myometrium (Fig. 4B).

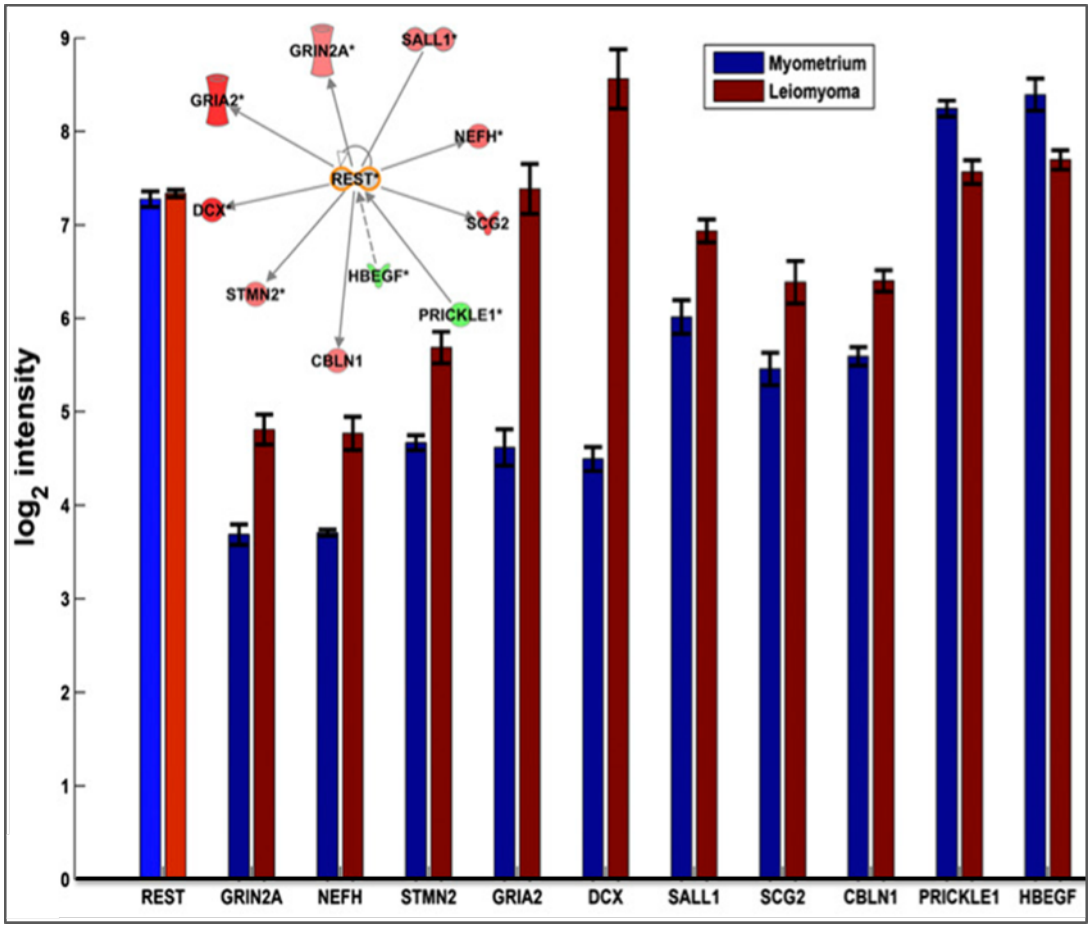

Figure 3: REST target genes are aberrantly expressed in uterine fibroids (Leiomyomas). Expression of known REST target genes in uterine fibroids from dataset GSE13319, pathway analysis (using IPA; Ingenuity Systems). Myometrium (blue), matched fibroids (red).

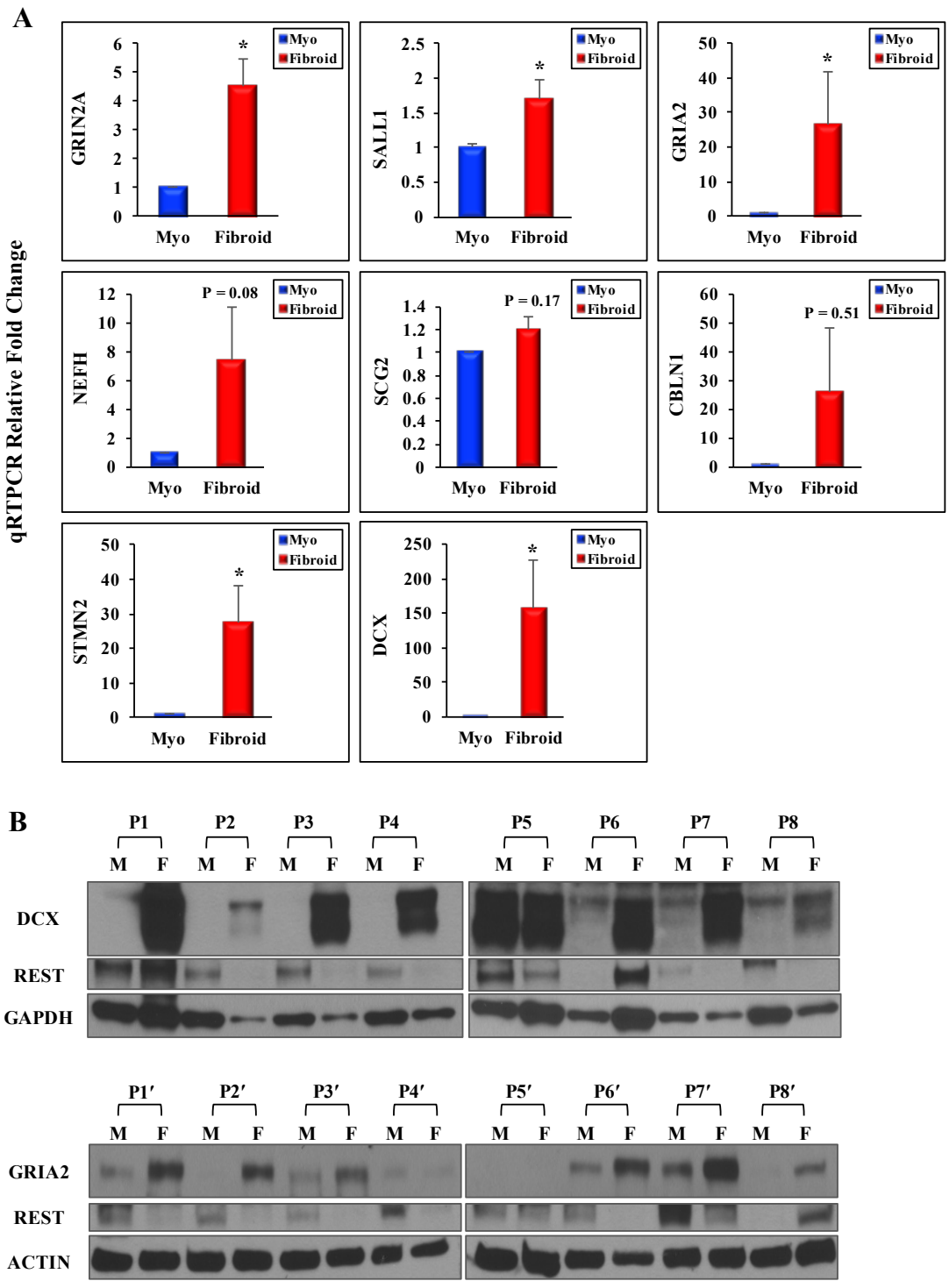

Figure 4: Expression of REST regulated genes are increased in patient fibroid samples. (A) TaqMan qRT-PCR analysis of REST target genes *SCG2, GRIA2, NEFH, SALL1, GRIN2A, STMN2, DCX and CBLN1* expression in 8-12 pairs of matched normal myometrial and fibroid samples. (B) Western blot analysis of protein extracts from paient (P1–P8) samples comparing DCX, GRIA2 and REST expression in normal myometrium to matched fibroid samples. GAPDH and β-actin were used as protein loading control. Error bars indicate ± SD, *P < 0.05. Statistical analyses were performed by Mann-Whitney U Test.

2. Loss of REST Leads to Dysregulation of Target Genes in Primary Myometrial Cells.

Next, we tested whether the aberrant expression of these REST target genes is actually due to the loss of REST in fibroids. To assess this problem, we silenced REST using siRNA (siREST) in primary myometrial SMCs cultured in vitro. Interestingly, we found *NEFH*, *GRIN2A* and *STMN2* to be expressed in the myometrial cells at higher levels in the absence of REST. Furthermore, *SCG2* and *SALL1* levels were slightly decreased in siREST treated cells and *DCX, GRIA2* and *CBLN1* levels were undetectable in cultured myometrial cells (Fig. 5). Our data suggests the loss of REST contributes to aberrant expression of a number of target genes in uterine fibroids.

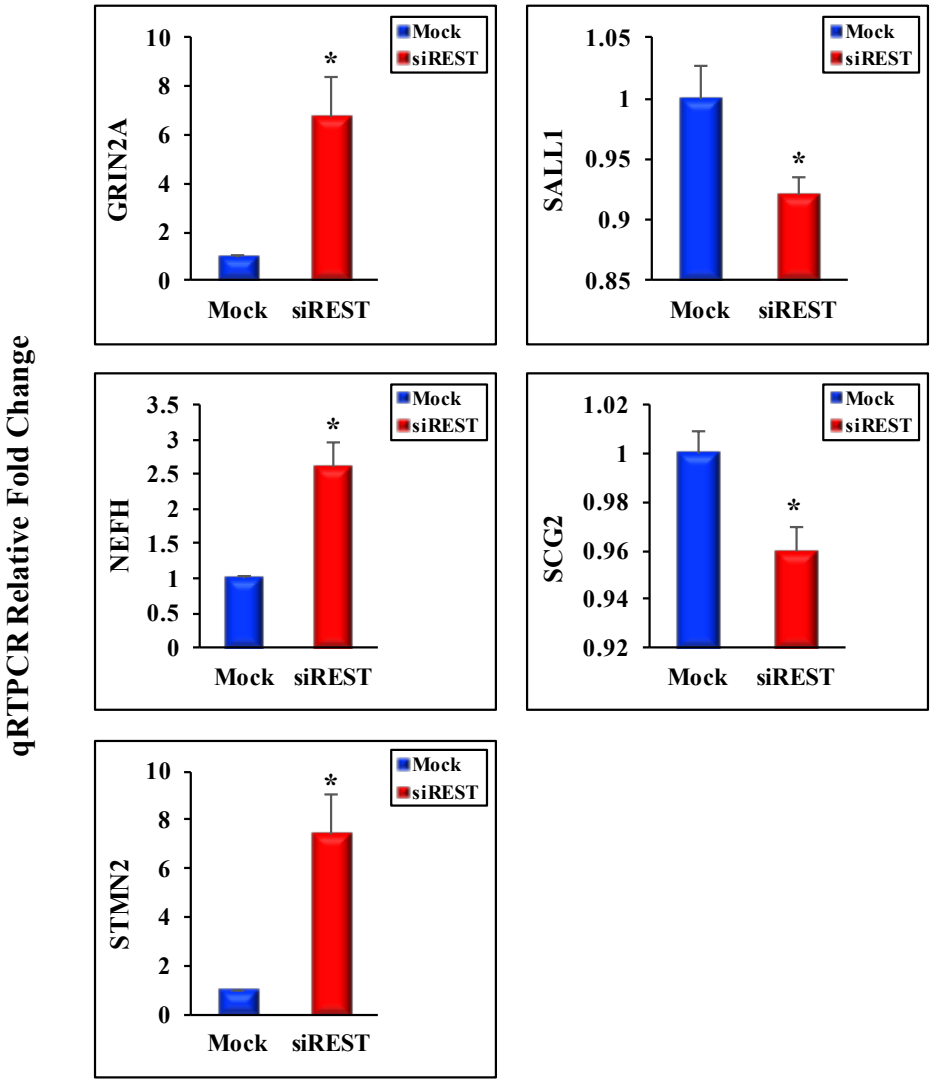

Figure 5: Expression of REST target genes is increased in REST silenced cells. TaqMan qRTPCR analysis of expression of *SCG2, NEFH, SALL1, GRIN2A, STMN2* in myometrial SMCs silenced with siREST and mock silenced cells (n= 3-4) for 48 h. (*DCX, CBLN1* and *GRIA2* were not detectable in cultured myometrial cells by qRTPCR). Error bars indicate ± SD, *P < 0.05. Statistical analyses were performed by Mann-Whitney U Test.

IV. Discussion

The mechanism of pathogenesis of uterine fibroids is currently unknown. Here we provide new insight on the effect of the degradation of tumor suppressor REST in fibroids on downstream target genes. We offer evidence that the loss of REST in primary myometrial cells leads to aberrant expression of many of these genes. Therefore, providing further information on the role of REST in fibroids. In our patient samples, we found most REST target genes to be aberrantly expressed in fibroid samples similar to the GSE database, however a larger and more diverse sample size may be needed to see a significant change in *CBLNI, NEFH, GRIA2* and *SCG2* expression levels. In our study, we have showed for the first time, the knockdown of REST in myometrial SMCs leads to a significant increase in *NEFH*, *GRIN2A* and *STMN2* genes. Additionally, *SALL1* and *SCG2* were slightly decreased in REST silenced cells, interestingly they were also among the least aberrantly expressed genes in our patient samples. Therefore, silencing REST in culture with siRNA may not be efficient enough in knocking down REST to see a change in these genes and a more stable short hairpin RNA (shRNA) for silencing or REST knockout mouse model may be needed. Furthermore, in our cell culture experiment we were not able to detect *DCX, GRIA2* and *CBLN1* mRNA levels via the sensitive technique of RT-PCR, suggesting the conditions of in vitro cell culture influences the expression of these genes and is not a suitable method for their study. In addition, DCX is a marker of immature neurons and therefore its expression could be lost in culture due to cell differentiation [221]. Although our results show a connection between loss of REST and its target genes in cultured SMCs, our model system is limited and additional experiments in an in vivo model is needed to further understand the regulation of these genes by REST in uterine fibroids.

REST is known to bind to the RE-1 binding site/ neuron-restrictive silencer element (RE-1/NRSE) and repress neuronal gene expression in non-neuronal cells [212]. Many of the REST target genes found to be overexpressed in uterine fibroids are neuronal genes and have important functions in the nervous system, including termination of cell-fate during neuronal development, maintaining synapses and stabilizing microtubules [222-228]. The aberrant expression of DCX in fibroids caught our interest, because in the adult brain it is expressed in immature neurons and is used as a marker for neuronal precursor cells [221]. It is thought uterine remodeling and its return to a basal state may be due to the presence of adult progenitor cells in uterine layers and the dysregulation of these cells may lead to fibroid formation [229]. However, further studies are needed to fully understand the possible role of these stem cells in uterine fibroid pathogenesis. The presence of the neuronal progenitor marker DCX, at high levels in uterine fibroids, may be a potential tool for recognition and isolation of these cells. This would allow for a more detailed look at these stem cells and to better understand their function in fibroid etiology.

Our findings have shown the loss of REST expression in uterine fibroids is responsible for dysregulation of a number of target genes. These genes could be important as biomarkers or drug targets and further studies are needed to fully understand their role in fibroid pathogenesis. Future studies using our conditional Rest knockout (Rest fl/fl – CaBP9K-Cre) mice will determine the regulation and functions of neuronal specific REST target genes aberrantly expressed in uterine fibroids.

Chapter III: Regulation of MicroRNA-29 by REST Controls ADAM12 and EGF Signaling in Uterine Fibroids.

I. Abstract

Uterine fibroids are benign smooth muscle cell tumors of the myometrium. These tumors pose a significant burden on patients with symptoms of pelvic pain, uterine bleeding, and frequent miscarriages; costing the US economy an estimated $34 billion annually. Currently there are no long-term drug treatments for fibroids that will leave fertility intact, mainly because of their unknown pathogenesis. One of the key characteristics of uterine fibroids is excessive deposition and reorganization of extracellular matrix (ECM). We found that ADAM12, an enzyme that is known to cleave ECM proteins, activate epidermal growth factor (EGF) signaling and promote tumorigenesis, is upregulated in fibroids. Interestingly, analysis of Ingenuity ® pathways in uterine fibroids showed a link between ADAM12 and the tumor suppressor REST, which is lost in fibroid tumors, through miR-29. In order to understand the link between REST, ADAM12 and miR-29, we silenced REST in primary myometrial cells and found a decrease in miR-29 gene expression and an increase in ADAM12 protein and EGF receptor (EGFR) phosphorylation levels. Furthermore, we found inhibiting miR-29 in myometrial cells results in an increase in ADAM12 expression and EGF pathway activation. Conversely, the opposite effects were seen when leiomyoma cells were treated with miR-29 mimics. In conclusion, we report a novel druggable pathway that links the loss of REST, down regulation of miR-29, increased ADAM12 expression, and the activation of EGFR in uterine fibroid pathogenesis.

II. Introduction

Uterine fibroids, also known as leiomyomas, are benign smooth muscle cell tumors of the myometrium. They are the most common tumor of the female reproductive tract with a cumulative incidence of greater than 80% among black women and 70% among white women by age 50 [1]. They account for the most common indication for hysterectomy and are estimated to cost the US greater than $34 billion on an annual basis [3]. Patients with uterine fibroids are known to suffer from abnormal bleeding, anemia, pain, pressure, incontinence and recurrent miscarriages. Despite the common occurrence of these tumors, currently there is lack of a cost effective and long-term treatment option which will leave fertility intact. The main reason for this absence is due to the unknown pathogenesis of uterine fibroids [2, 32]. Therefore, there is a clear need for increased research in this area to further our knowledge on the mechanisms that trigger the formation and support the growth of uterine fibroids to help us in developing better and more effective treatment options.

One of the pathways known to be important for the pathogenesis of uterine fibroids is the phosphatidylinositol-3 kinase (PI3K)/Akt signaling pathway, which activates the mammalian target of rapamycin (mTOR) leading to cell growth and proliferation [140, 141]. Recently our lab has shown an upstream regulator of this pathway, the RE1 silencing transcription factor/neuron-restrictive silencing factor (REST/NRSF), to be lost in uterine fibroids [142]. REST is a transcriptional regulator best known for its role in suppressing neuronal gene expression in non-neural tissue [212]. Our lab has shown the importance of REST in fibroid formation through our REST conditional knockout (cKO) mouse model, where in the absence of REST the mouse uteri are enlarged and have excess collagen deposition (Koohestani, McWilliams, unpublished data). How the loss of REST is contributing to the formation of

fibroids is still largely unknown. One of the main characteristics of uterine fibroids is the excess deposition and reorganization of extracellular matrix (ECM) [182]. Analysis of gene expression datasets from GEO database (GSE13319) shows an upregulation of a member of the A-Disintegrin and Metalloprotease family of matrix modifying enzymes, ADAM12, in fibroids. ADAM12 is known to cleave ECM proteins, activate epidermal growth factor (EGF) and Insulin-like growth factor (IGF) signaling and promote tumorigenesis [193]. Interestingly, Ingenuity® pathway analyses of gene expression datasets for uterine fibroids indicate that the loss of REST and increase of ADAM12 in fibroids may be linked through a third player, MicroRNA29 (miR-29). MicroRNAs are small non-coding RNAs which are known to inhibit the transcription and translation of their target genes by binding to specific sites in their 3'UTR [98]. The miR-29 family has a diverse pool of targets and is known to play an important role in many cancers and fibrotic diseases [109]. Research by other groups has shown the 3' UTR of ADAM12 to be targeted by miR-29 [230]. In addition, REST has been shown to bind to the repressor element-1 (RE-1) site upstream of the miR-29 promoter [231]. Unlike the vast majority of gene targets known to be repressed by REST, miR-29 is downregulated in fibroid tumors [108] where REST expression is lost [142], suggesting a novel transcriptional activator role for REST. Based on the data presented below, we show for the first time that the loss of REST in myometrial cells leads to a decrease in miR-29 levels along with an increase in ADAM12 expression. In addition, the overexpression of ADAM12 activates a number of pathways in primary myometrial cells, which have important roles in cell survival and tumor growth. Our data support a novel pathway connecting the loss of REST and increase in ADAM12 in fibroids mediated by the loss of miR-29 expression (Fig. 6).

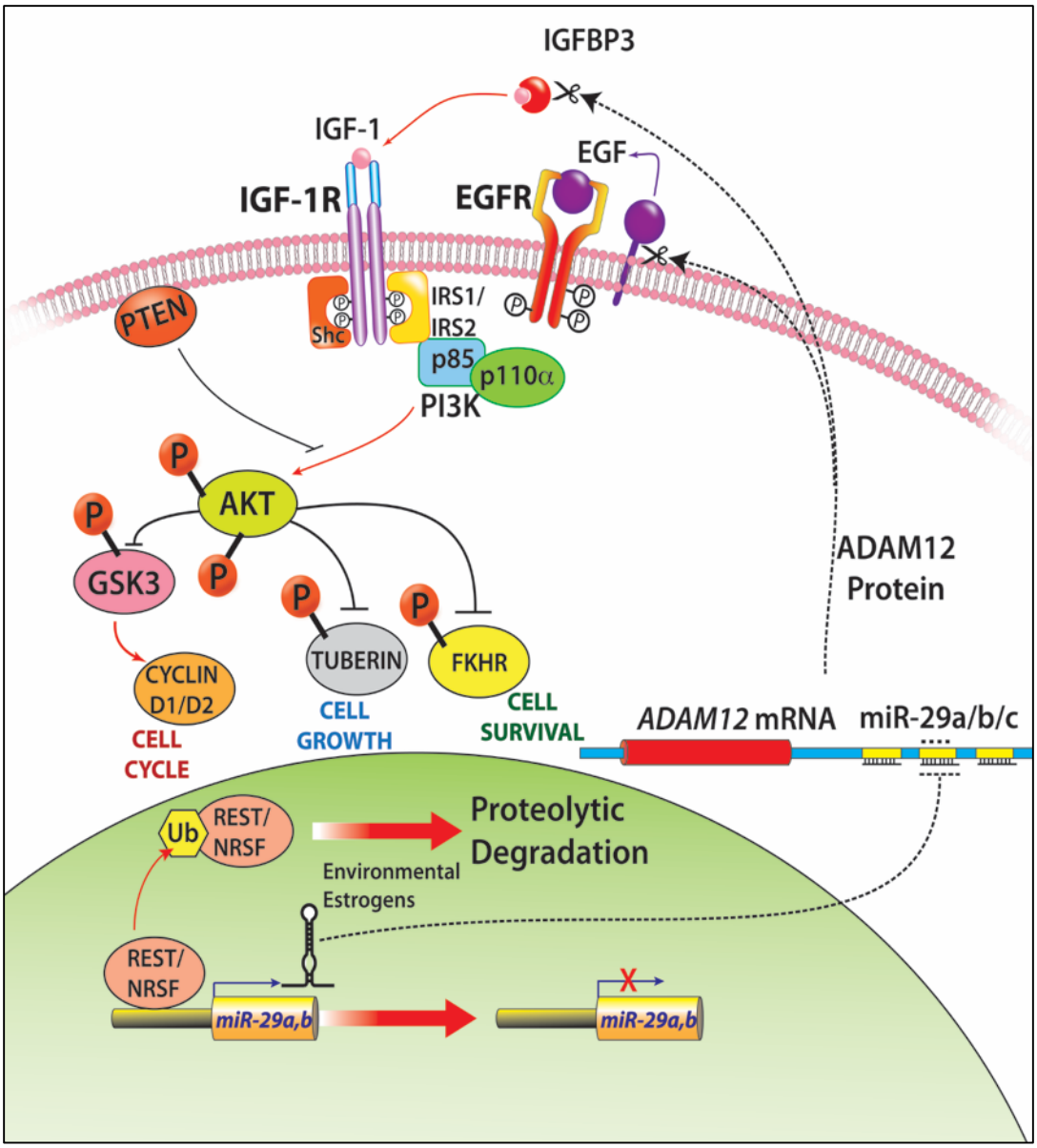

Figure 6: Working model representing the loss of REST and miR-29 leading to loss of inhibition on ADAM12 and downstream activation of signaling pathways in the myometrium.

III. Results

1. ADAM12 is Aberrantly Expressed in Uterine Fibroids

In an effort to understand the pathogenesis of uterine fibroids, our lab recently discovered a tumor suppressor, REST, to be lost in these tumors. To learn further about the role of REST in fibroid formation, we looked at GEO database, dataset GSE13319, for expression of known REST target genes in fibroids. During our search we found the metalloproteinase, *ADAM12*, a member of the greater ADAM family of enzymes to be highly upregulated in the tumors (Fig. 7A). ADAM12 has been associated with the pathogenesis of a number of cancer and fibrotic diseases [199-202]. Next, we used TaqMan RT-PCR to confirm the expression of *ADAM12* mRNA in our patient samples. Similar to the GEO database we found a significant increase in *ADAM12* levels in tumor samples compared to unaffected myometrium (Fig. 7B). Western blot analysis also verified this aberrant expression at the protein level where we found an increase in ADAM12 in 4 out of 5 patient fibroid samples (Fig. 7C). As part of the ADAM family of metalloproteinases, ADAM12 may have functional overlap with other catalytically active members of the family [232]. Therefore, we analyzed the GEO database, dataset GSE13319, for the expression pattern of all available ADAM families. Interestingly, our results show *ADAM12* to be the most highly dysregulated ADAM in uterine fibroids (Fig. 8). The aberrant expression of ADAM12 is a profile similar to what is seen in many other cancers, suggesting this enzyme could be playing a role in the pathogenesis of uterine fibroids [193].

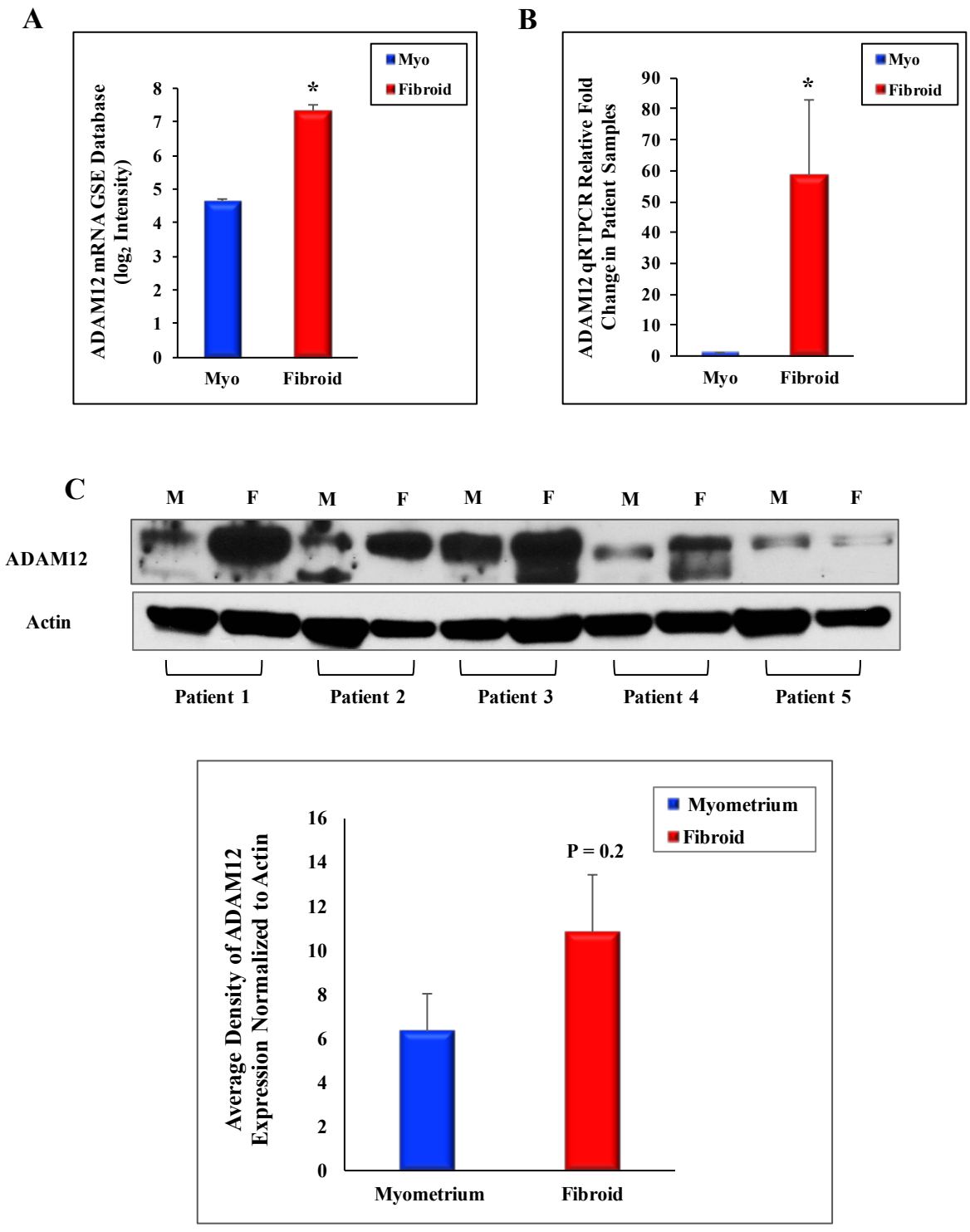

Figure 7: ADAM12 is overexpressed in uterine fibroids. (A) Expression of *ADAM12* in matched normal myometrium and uterine fibroids from dataset GSE13319. (B) TaqMan qRT-PCR analysis of *ADAM12* expression in 11 pairs of matched normal myometrial and fibroid samples. (C) Western blot and densitometric analysis of Western blots of protein extracts from patient samples (1-5) comparing ADAM12 expression in normal myometrium to matched fibroid samples. β-actin was used as protein loading control. Error bars indicate ± SD, *P < 0.05. Statistical analyses were performed by Mann-Whitney U Test.

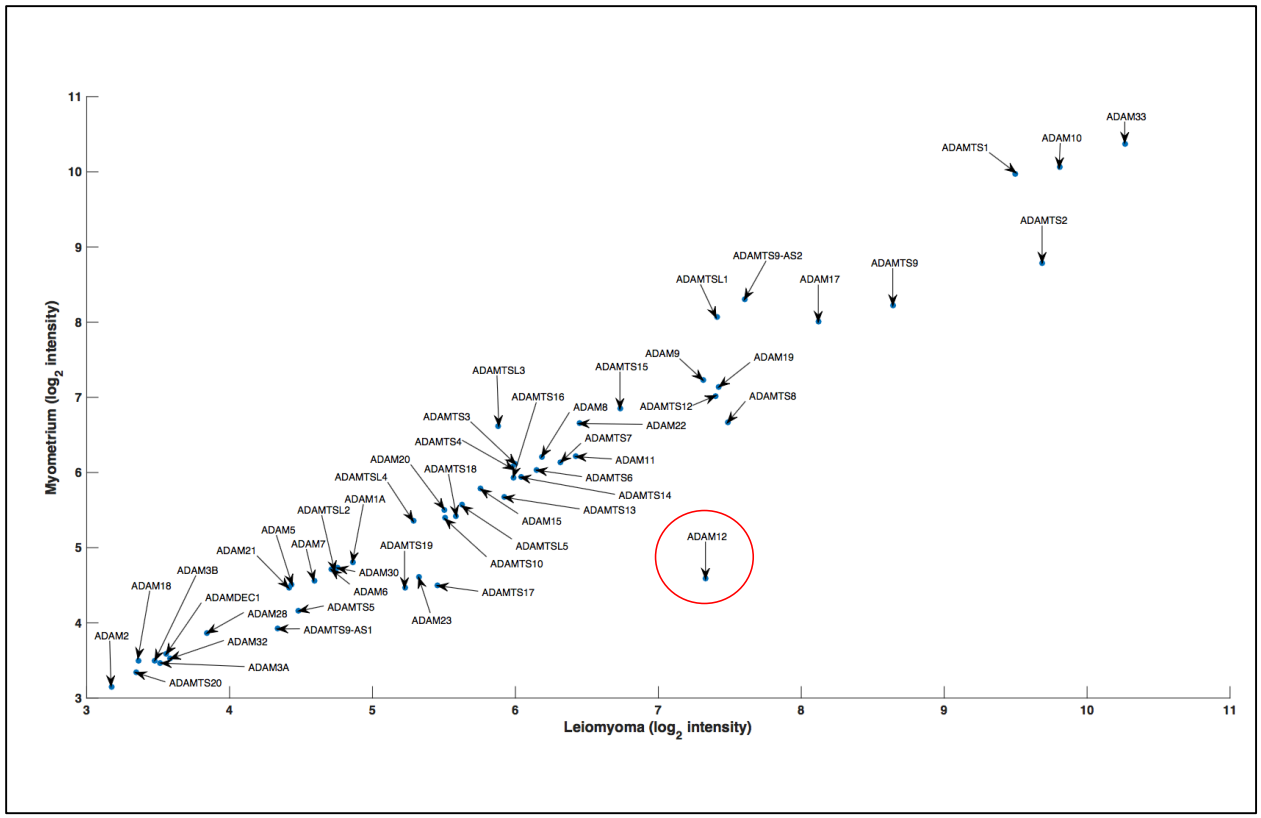

Figure 8: *ADAM12* is the most highly dysregulated member of the ADAM family in uterine fibroids. Expression of ADAMs in matched normal myometrium and uterine fibroids from dataset GSE13319. ADAM12 is circled in red.

2. ADAM12-L is the Predominant Isoform in Uterine Fibroids

ADAM12 is present in two isoforms, a transmembrane (ADAM12-L) and a secreted form (ADAM12-S). The two isoforms have been reported to process different substrates and have distinct functions in several pathological diseases. ADAM12-S cleaves insulin-like growth factor-binding protein 3 (IGFBP3), IGFBP5 and ECM substrates. ADAM12-L on the other hand is known to shed membrane bound ligands HBEGF, EGF, betacellulin, Delta-like 1 and placental leucine aminopeptidase [233]. In human breast tumor cells, ADAM12-S has been reported to be exclusively responsible for enhancing tumor cell migration and invasion in vitro and increase rates of metastasis in vivo [234]. Shao and colleagues confirmed this function of ADAM12-S in lung cancer cells, where they found ADAM12-L to promote proliferation and ADAM12-S to promote invasion and metastasis [235]. Based on the differences between the ADAM12 isoforms, we became interested in knowing if a specific form of ADAM12 is dominantly expressed in fibroids. In this study, we analyzed matched patient fibroid and myometrial samples with Taqman RT-PCR, and found *ADAM12-S* and *ADAM12-L* to be 30 fold and 54 fold increased, respectively, in tumors compared to normal myometrium (Fig. 9). Although both forms of *ADAM12* are significantly increased, *ADAM12-L* is the predominant isoform in uterine fibroids, based on comparing cycle numbers in qRT-PCR.

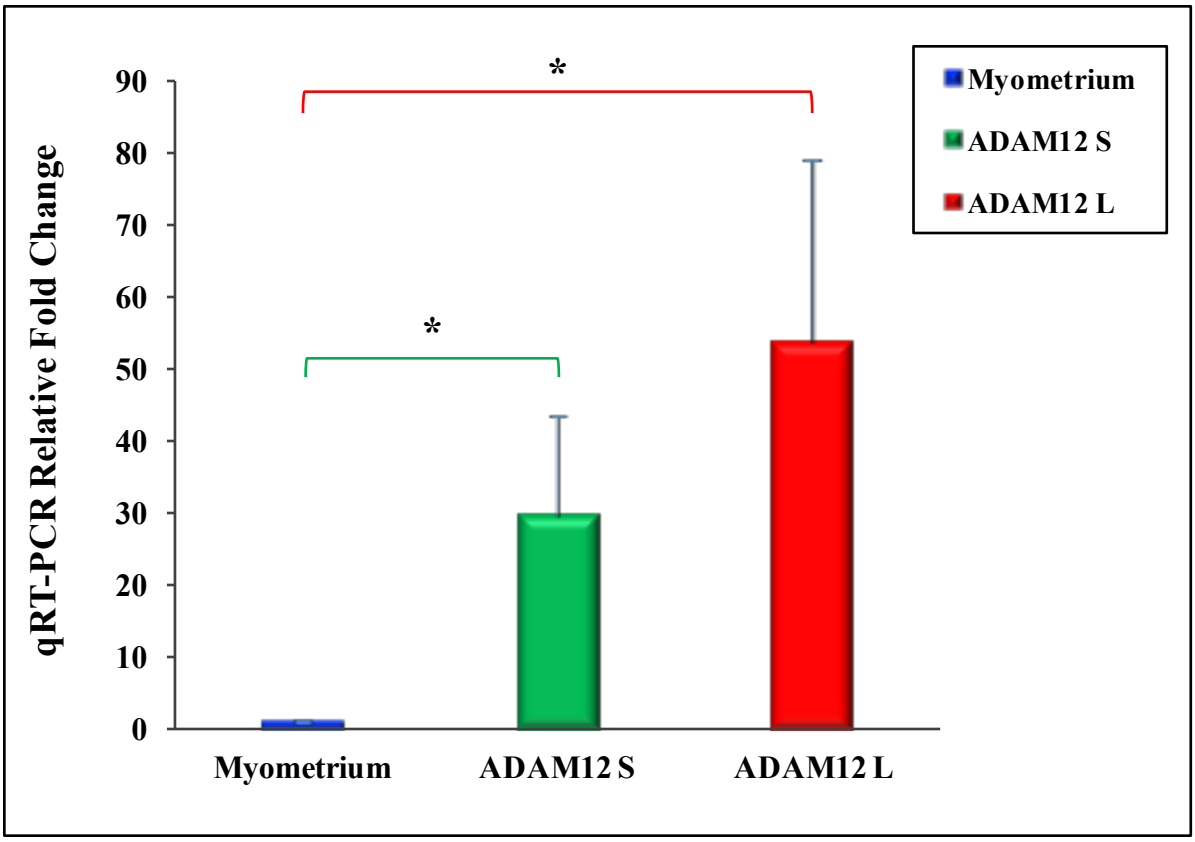

Figure 9: *ADAM12 L* is the predominant isoform expressed in uterine fibroids. TaqMan qRT-PCR analysis of two *ADAM12* isoforms, *ADAM12-S* and *ADAM1-L* expression in 10 pairs of matched normal myometrial and fibroid samples. Error bars indicate ± SD, *P < 0.05. Statistical analyses were performed by Mann-Whitney U Test.

3. Loss of REST Leads to higher levels of ADAM12

The high levels of ADAM12 in uterine fibroids alongside a loss of REST led us to test the possibility of a connection between the two. For this analysis, we knocked down REST in primary myometrial SMCs cultured in vitro using siREST. We found using TaqMan and Western blot analysis that *ADAM12* is indeed increased when REST is silenced in myometrial SMCs at the mRNA and protein levels (Fig. 10A, 10B). Furthermore, we found an increase in EGFR phosphorylation with Western blot in the REST silenced cells (Fig. 10B). In conclusion, our in vitro data supports for the first time the regulation of ADAM12 by REST in the uterus. Additionally, our data reveal a role for ADAM12 in activating EGFR signaling, a major tumor promoting pathway, as a novel mitogenic pathway in uterine fibroids.

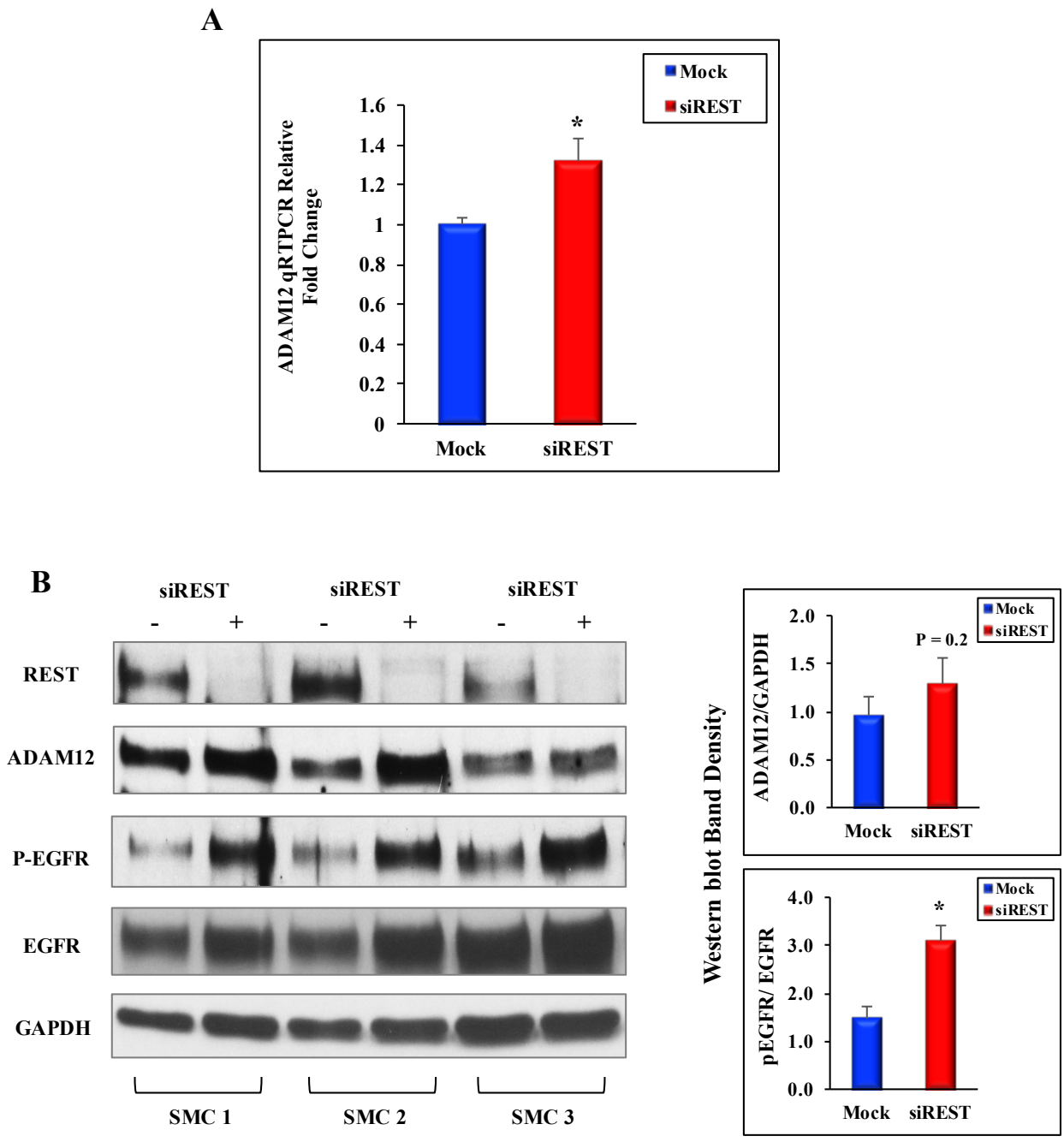

Figure 10: ADAM12 and EGFR phosphorylation is increased in REST silenced cells. (A) TaqMan qRT-PCR analysis of *ADAM12* expression in cultured myometrial SMCs silenced with siREST and mock silenced cells (n= 4) for 48 h. (B) Western blot and densitometric analysis of Western blots of protein extracts from myometrial SMCs silenced with siREST and mock silenced cells comparing ADAM12 expression and EGFR phosphorylation levels. GAPDH was used as protein loading control. Error bars indicate ± SD, *P < 0.05. Statistical analyses were performed by Mann-Whitney U Test (A) and Student's T-test (B).

4. REST Positively Regulates MiRNA29 Expression

Our results show a connection between the loss of REST and aberrant expression of ADAM12 in fibroids. We used Ingenuity® pathway analyses to further understand the putative mechanism of this regulation. Interestingly, we found REST and ADAM12 are suggested to be connected through miR-29. The miR-29 family in humans has four members; miR-29a and miR-29b-1 encoded by a gene on chr. 7q32.3 and miR-29b-2 and miR-29c which are encoded by a gene on chr. 1q32.3. The mature forms of miR-29b-1 and 29b-2 have identical sequences and are known as miR-29b [109]. Work by Qiang et al. has shown the down regulation of miR-29b in fibroids is essential for ECM accumulation and tumor maintenance [108]. Furthermore, Wu and colleagues have reported the presence of an RE1 element, a potential binding site for REST, upstream of the miR-29a and 29b-1 promoter [231]. Based on this information, we became interested in further understanding the relationship between REST and miR-29 in uterine fibroids. Initially, we analyzed expression levels of miR-29a and 29b in matched fibroid and myometrial samples using Taqman RT-PCR. We found miR-29a and 29b to be significantly down-regulated in fibroid samples (Fig. 11A) similar to previous reports by others [108, 110]. Next, we investigated the regulation of miR-29 by REST in uterine fibroids. Here we knocked down REST in primary myometrial SMCs and found a significant loss in miR-29a in addition to a decrease, albeit not statistically significant, in miR-29b ($P= 0.12$) expression levels upon the loss of REST (Fig. 11B). Furthermore, to more closely study the interaction between REST and miR-29, we performed chromatin immunoprecipitation assays (ChIP). Interestingly, we found decreased RNA polymerase II binding at the transcriptional start site of miR-29, which coincided with decreased REST association in fibroid samples (Fig. 11C). REST is a tumor suppressor,

known to repress transcription of neuronal genes in non-neural tissue [213]. However, our results support a novel role for REST as a positive regulator of miR-29 in uterine fibroids.

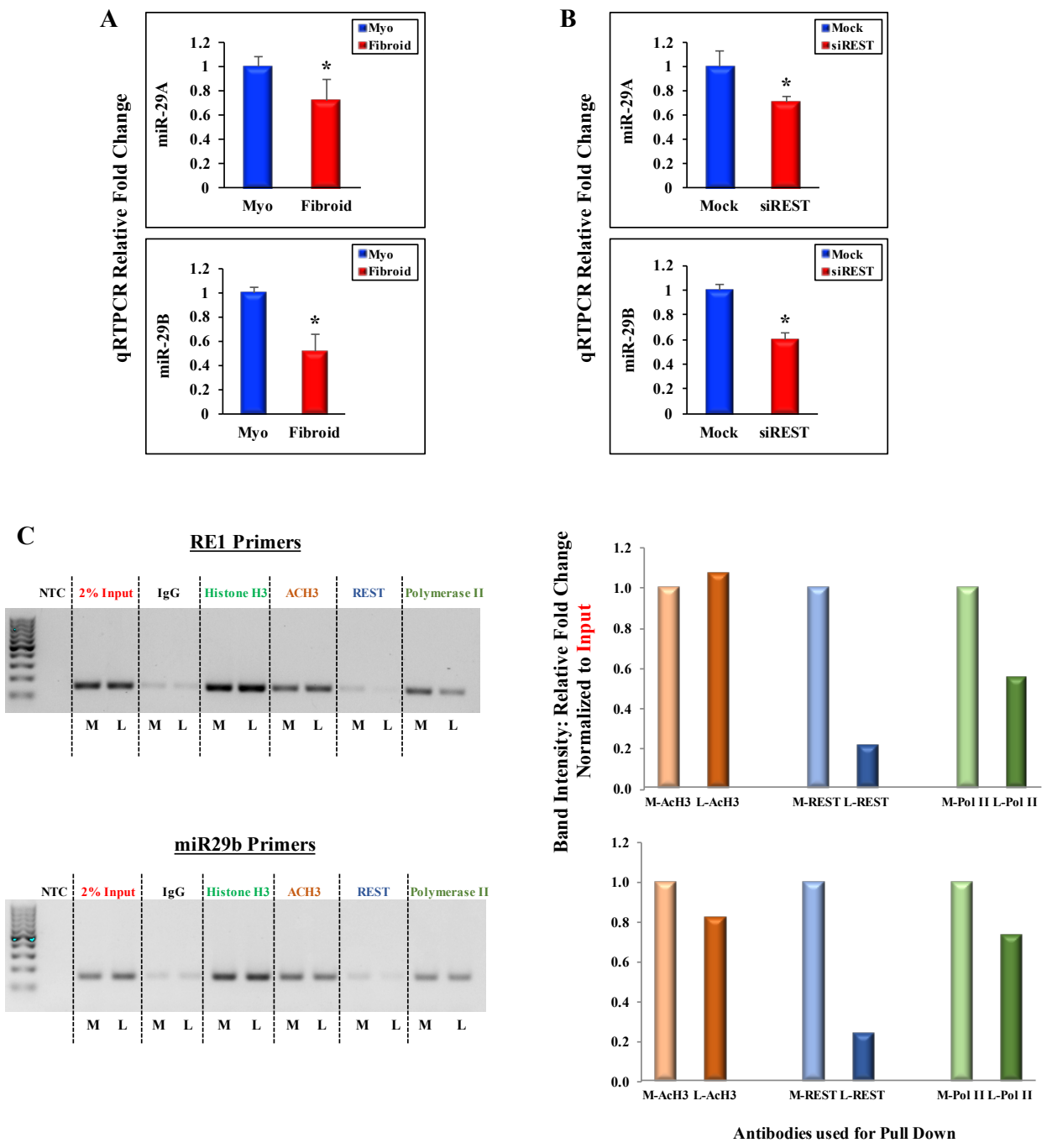

Figure 11: MircoRNA-29 is positively regulated by REST in the myometrium. (A) TaqMan qRT-PCR analysis of miR-29A (n=14) and 29B (n=17) expression in matched normal myometrial and fibroid samples. (B) TaqMan qRT-PCR analysis of miR-29A and 29B expression in cultured myometrial SMCs silenced with siREST and mock silenced cells (n= 7) for 48 h. (C) REST, acetylated histone H3 and RNA polymerase II were immunoprecipitated along with cross-linked chromatin, followed by amplification of miR-29 promoter and RE1 binding site in matched myometrial and leiomyoma SMCs (n=2). Error bars indicate ± SD, *P < 0.05. Statistical analyses were performed by Mann-Whitney U Test.

5. MiRNA29 Down-regulates ADAM12 Expression

Based on our results showing the loss of REST led to the aberrant expression of ADAM12 and miR-29 in uterine fibroids, we became interested in further investigating the connection between miR-29 and ADAM12 in these tumors. MicroRNAs are known to regulate their target genes by binding to their untranslated region (UTR) [96]. The two isoforms of ADAM12, ADAM12-S and ADAM12-L, have distinct 3′UTRs and miR-29 has been shown to specifically target ADAM12-L but not the secreted short form [230]. In order to test the relationship between miR29 and ADAM12 in fibroids, we used gain and loss of function approach to change miR-29 levels and measure its effect on ADAM12 expression. Here we transfected primary myometrial cells with miR29-a and 29b synthetic miRCURY LNA™ inhibitors targeting endogenous miR-29 for 48 hours and then measured mRNA levels of *ADAM12* and ECM components, collagens I and III, using Taqman RT-PCR. Interestingly, we found a significant increase in *ADAM12* and collagen expression levels with miR-29a inhibition compared to negative control, but no significant change was detected with miR-29b inhibition (Fig. 12A). Next, we transfected primary fibroid cells with miR-29a and 29b synthetic miRCURY LNA™ mimics designed to simulate endogenous mature microRNAs. Our results show a significant decrease in *ADAM12* and collagens I and III mRNA expression levels with miR-29a and 29b mimic treatment compared to negative control (Fig. 12B). These results show *ADAM12* to be negatively regulated by miR-29 in uterine SMCs. The results also indicate redundancy of miR-29 a/b isoforms in the regulation of ADAM12 expression. Taken together, the loss of miR-29 due to loss of regulation by REST in uterine fibroids could be causing the aberrant expression of ADAM12.

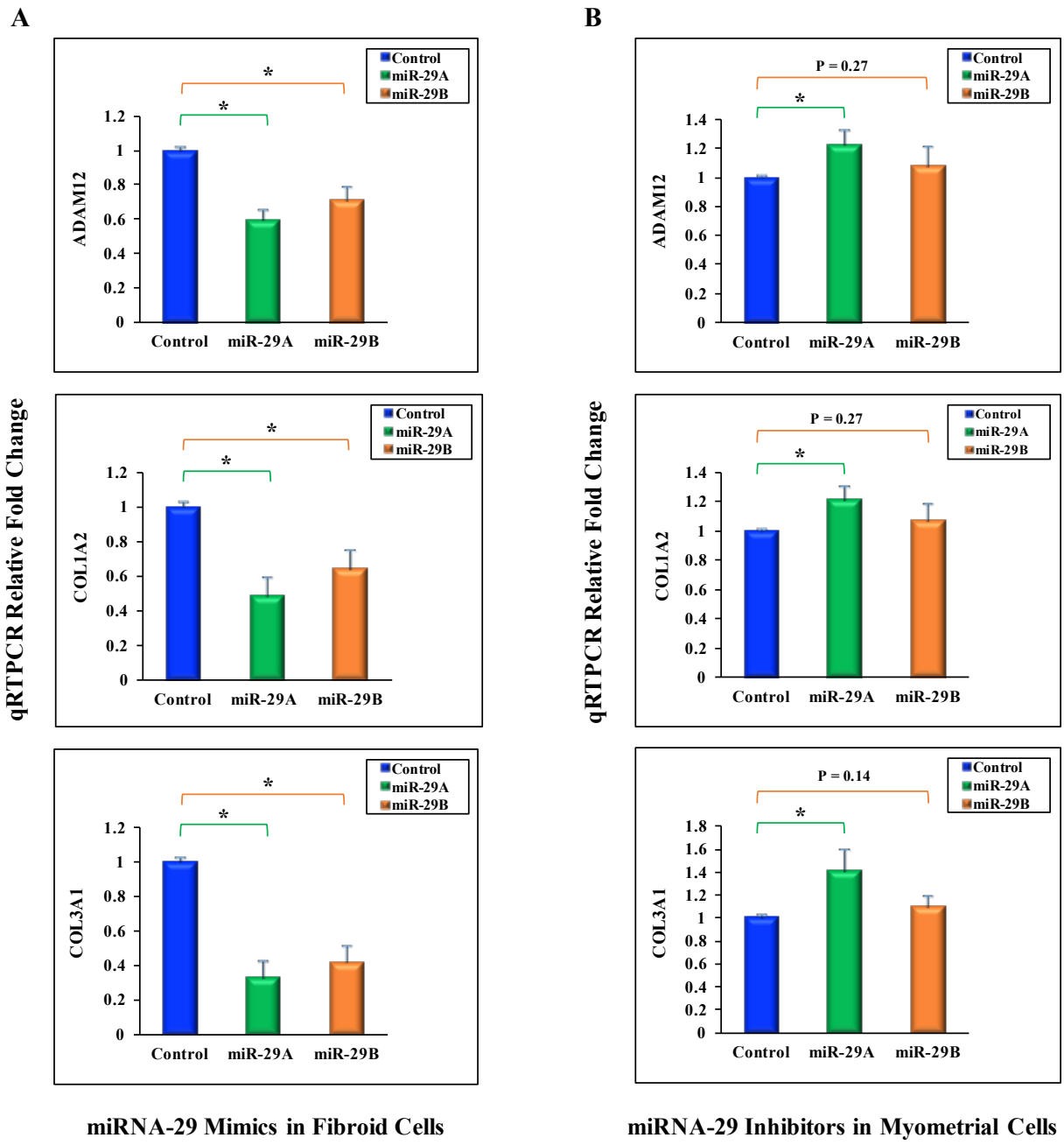

Figure 12: MicroRNA-29 negatively regulates the expression of ADAM12 and collagens in uterine fibroids. TaqMan qRT-PCR analysis of *ADAM12, COL1A2* and *COL3A1* expression levels (A) in cultured primary fibroid SMCs treated with miR-29A and 29B mimics (50 nm) and (B) in cultured primary myometrial SMCs treated with miR-29A and 29B inhibitors (100 nm) or negative control (n= 6) for 48 h. Error bars indicate ± SD, *P < 0.05. Statistical analyses were performed by Mann-Whitney U Test.

6. ADAM12 Activates the EGFR Pathway in Primary Myometrial Cells

The EGFR pathway is implicated in the pathogenesis of numerous cancers. It is involved in tumor growth, cell proliferation and tissue homeostasis [172]. There has been conflicting information on expression of EGFR in myometrial and fibroid samples, and the information on the activity of this pathway in fibroids is missing [178, 180]. ADAM12 has a role in cleaving EGFR ligand, heparin-binding EGF (HB-EGF), therefore making it available to bind to its receptor and lead to downstream pathway activation [207]. When we silenced REST in primary myometrial SMCs we saw an increase in ADAM12 and downstream increase in EGFR phosphorylation (Fig. 10B). In order to understand if this increase in EGFR phosphorylation is due to increased ADAM12 levels we overexpressed ADAM12 in primary myometrial SMCs. For this study, we designed an adenoviral vector bearing the ADAM12-L cDNA with a V5 tag. Using this constructed plasmid, we were able to successfully overexpress ADAM12 in 293A cell line (Fig. 13A). Upon transduction of primary myometrial SMCs with the adenovirus for 48 hours in 2% FBS media we found an increase in ADAM12 and downstream EGFR phosphorylation levels with Western blot (Fig 13B). In conclusion, we have shown due to the loss of REST there is an increase in ADAM12 activity leading to increased EGFR activation. This activation can be important in tumor growth and cell proliferation.

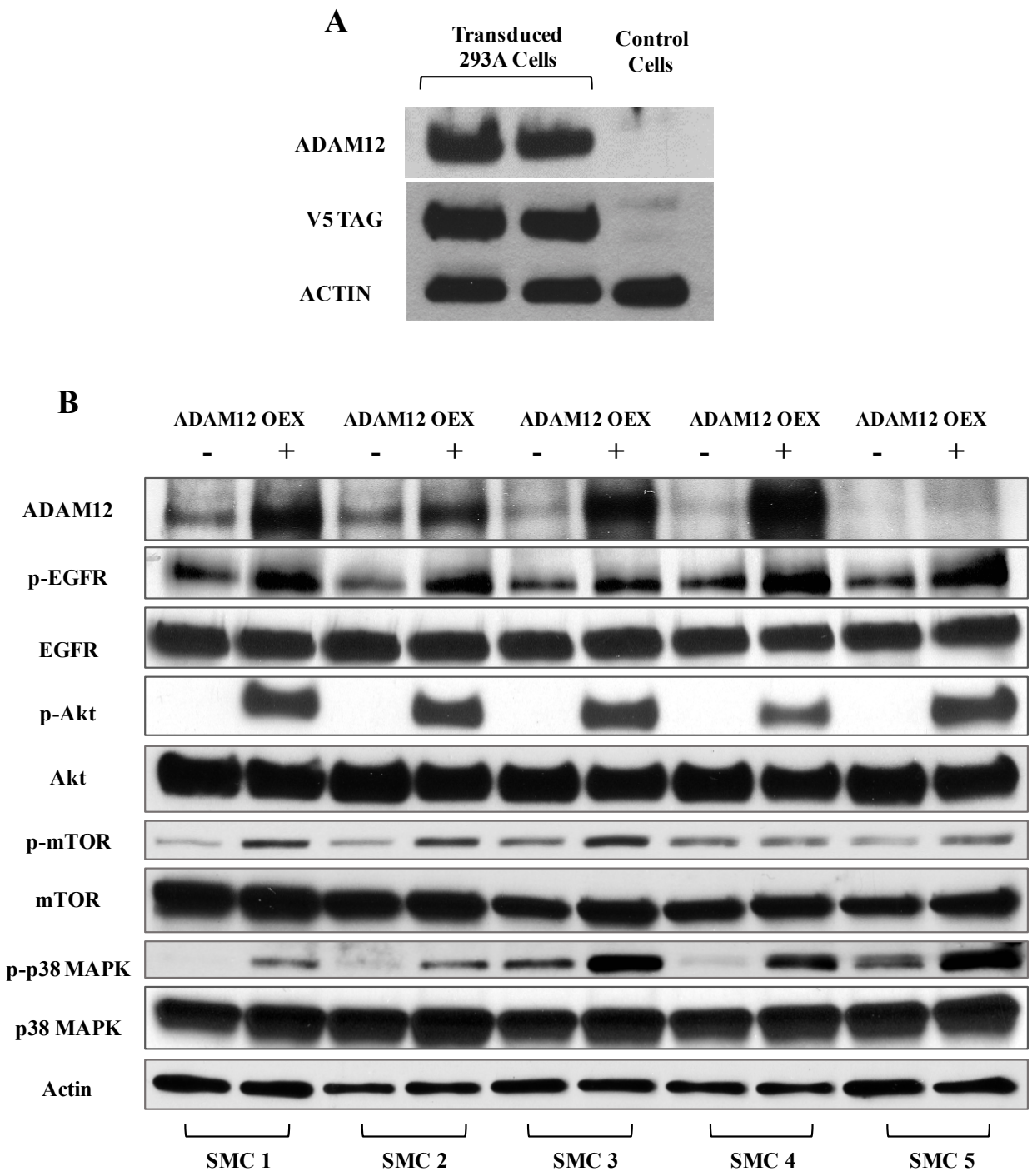

Figure 13: ADAM12 overexpression activates tumorigenic pathways in myometrial cells. Western blot analysis of protein extracts from cells treated with adenoviral plasmids containing *ADAM12* cDNA (ADAM12 OEX) or mock transduced for 48 h in (A) 293A cells comparing ADAM12 and the adenoviral V5 tag expression levels and in (B) myometrial SMCs comparing ADAM12 expression, EGFR, Akt, mTOR and p38 MAPK phosphorylation levels. β-actin was used as protein loading control.

7. ADAM12 Overexpression Activates Multiple Signaling Pathways.

One of the most highly dysregulated pathways in uterine fibroids is the PI3K-Akt-mTOR pathway [140]. The activation of this pathway leads to cell survival, proliferation and growth [138]. Mitogen-activated protein kinase (MAPK) is another pathway involved in regulation of cell survival, proliferation and migration. The MAPK family is composed of subfamilies; extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 MAPK. Inflammatory cytokines and stress are known to activate p38 MAPK which can lead to autoimmune diseases and cancers [236, 237]. Previous work has shown the aberrant activation of MAPK/ERK pathway in uterine fibroids by 17beta-estradiol treatment [238]. However, little is known about the role of p38 MAPK in fibroid etiology. Observing the effect of ADAM12 on EGFR activation we became interested in looking at additional tumorigenic pathways in fibroids to help further our understanding of the role of ADAM12 in fibroid pathogenesis. Therefore, we tested whether ADAM12 overexpression could activate PI3K-Akt-mTOR and p38 MAPRK pathways. For this experiment, we cultured primary myometrial SMCs *in vitro* and treated them with adenovirus harboring *ADAM12-L* cDNA for 48 hours. Interestingly, when analyzed by Western blot we found a robust increase in Akt, mTOR and p38 phosphorylation in myometrial cells with ADAM12 overexpression (Fig. 13B). Our results suggest ADAM12 to have a wider role in aberrant activation of PI3K-Akt-mTOR and p38 MAPK pathways in uterine fibroids. Understanding the regulation of these pathways might help in development of a combination inhibitor of signal transduction for achievement of therapeutic efficiency and specificity for uterine fibroids.

IV. Discussion

Uterine fibroids are very common and cause great morbidity in patients effected. Currently the mechanisms involved in their pathogenesis is largely unknown and therefore there is sparse resources available to design specific therapies for long term treatment of fibroids [32]. The removal of the uterus and consequently, termination of fertility of women, through hysterectomy is the main stay for uterine fibroid treatment [49]. Identification of specific molecules which are important in fibroid formation could enable development of novel therapeutic options and help improve quality of life for patients.

Our lab recently discovered the tumor suppressor REST to be lost in uterine fibroids [142]. However, little is known about the role of REST in the pathogenesis of these tumors. The purpose of our study was to further our understanding on the effect of REST degradation on downstream target genes in fibroids and to find important players in their pathogenesis. In our search, we found *ADAM12* to be aberrantly expressed in fibroid samples in the GEO database. In addition, we found ADAM12 to be connected to REST, putatively through miR-29. We hypothesized the loss of REST leads to decreased miR-29 and loss of inhibition on ADAM12, allowing for aberrant expression of this enzyme leading to downstream activation of critical pathways in tumor growth.

ADAM12 is a disintegrin and metalloproteinase enzyme known to remodel ECM and shed growth factor ligands [197, 207]. In breast cancers, ADAM12 levels are highly dysregulated, and its urinary output directly correlates with cancer progression [199]. We have provided evidence that the aberrant expression of ADAM12 in myometrial SMCs leads to downstream activation of EGFR, PI3K-Akt-mTOR and p38 MAPK pathways. These pathways are known to be important

in cell growth, proliferation and tumorigenesis [172, 237, 239]. Therefore, our data support for the first time a role for ADAM12 in uterine fibroid pathogenesis.

The importance of miR-29 downregulation in fibroid formation has been previously shown using a xenograft model by Qiang et al. [108]. In addition, in breast cancer cells, Duhachek-Muggy and colleagues have shown miR-29 to target ADAM12 3′UTR and inhibit its expression [230]. However, little information is available on the connection between miR-29 and ADAM12 in fibroids. Treating primary myometrial and fibroid cells with miR-29 inhibitors and mimics, we were able to show increased miR-29 in fibroid cells decreased the levels of ADAM12 and miR-29 inhibitors in myometrial cells increased ADAM12 levels. These data support our hypothesis that ADAM12 is overexpressed in fibroids by the loss of miR-29.

REST regulates its target genes by binding to the RE-1 binding site/ neuron-restrictive silencer element (RE-1/NRSE) [212]. Previous work has found miR-29 promoter to be in close vicinity of the RE-1 site, suggesting miR-29 could be regulated by REST [231]. We provide evidence for this connection through knockdown of REST in primary myometrial cells, where we found a decrease in miR-29 expression. REST is commonly known as a master negative regulator of genes [240]. Our data however suggest a novel role for REST as a positive regulator of miR-29 expression in the myometrium. REST has two independent repression domains located at the N and C terminal domains [241]. Work by Abramovitz et al. has shown the N-terminal domain of REST to induce the activity of the glucocorticoid receptor, while the C-terminal domain and full-length REST inhibit this activity [242]. Similar roles in activation of gene transcription has been shown by REST splice variant, REST4, which lacks the C-terminal domain of REST and is exclusively expressed in neural tissue [243, 244]. Although these studies support a dual role for REST in activation and repression of gene expression, their observation of induction in gene

activity has been limited to the N-terminal domain of REST. However, our results suggest the full length form of REST to be involved in positive regulation of miR-29a/b expression in uterine fibroids. Through knockdown of full length REST by siRNA, we have shown a loss in REST expression leads to decreased levels of miR29a/b in myometrial SMCs. Furthermore, our ChIP experiment showed decreased RNA polymerase II binding at the transcriptional start site of miR-29, which coincided with decreased REST association in fibroid samples. Further research is needed to understand the molecular switching of REST between a repressor and an activator.

The main limitation of our study is the exclusive use of *in vitro* systems. We have attempted to make sure our data are representative of uterine fibroids in vivo, by only using primary cells derived from human myometrial and fibroid samples in our studies. Although our model system has been limited, our data provide strong preliminary information which can be further examined in future studies in animal models and in vivo systems. Our lab has recently developed a conditional knockout mouse model where REST is specifically knocked out in the myometrium. This will be an invaluable tool to further examine the connection between REST, miR-29 and ADAM12 in vivo.

To help fully understand the importance of ADAM12 in fibroid pathogenesis, we will need to analyze the effect of its inhibition or overexpression on fibroid tumor growth and maintenance in vivo. A xenograft model where human tissue is placed under the renal capsule of immune-deficient mice, allows for in vivo treatment of fibroids and myometrial samples with ADAM12 and its inhibitors. This will allow us to further examine ADAM12 as a possible drug target for fibroid treatment. Currently, synthetic inhibitors of ADAM12 are being studied as potential treatment options for different diseases. KB-R7785 is one of these inhibitors which blocks the catalytic activity of ADAM12 in cardiac hypertrophy [207]. In addition, a more specific target

to the ADAM12 recombinant prodomain has been designed by Miller and colleagues to inhibit HB-EGF shedding in endometriosis [210]. A drug which specifically targets ADAM12 in the tissue of interest, is well-tolerated, and effectively inhibits its activity will have great potential for treatment of many diseases.

The field of uterine fibroids lacks an effective long-term treatment for its patients. Our data provide evidence for the first time a role for ADAM12 in fibroid pathogenesis through activation of downstream tumorigenic pathways. Identification of these important players in uterine fibroid etiology is the first step in the development of better treatment options to help decrease patient morbidity.

Chapter IV: Notch Pathway

I. Abstract

Uterine fibroids are the most prevalent reproductive tumors of the female reproductive tract. Currently the knowledge on the pathophysiology of these tumors is lacking. Due to this lack in knowledge, the long-term therapy options for fibroids are limited. Discovering pathways involved in fibroid etiology could aid in the development of new and effective drugs for uterine fibroid therapy. The Notch receptor is involved in cell contact dependent signaling and has important roles in cell survival, proliferation and differentiation. Currently, no role for the Notch pathway has been shown in uterine fibroids. In an attempt to discover new druggable pathways in uterine fibroids, we investigated the status of Notch pathway in these tumors. Using sections from matched myometrial and fibroid samples we found increased staining for cleaved, activated form of Notch receptor in the nucleus of fibroid cells. In addition, we saw an increase in Notch target genes *CCND1* and *ATF2* mRNA and Hes1protein levels in fibroid samples compared to matched myometrium. Furthermore, our data indicate a role for ADAM12, a disintegrin and metalloproteinase, in Notch activation. Using adenovirus harboring *ADAM12* cDNA, we overexpressed ADAM12 in primary myometrial cells and found an increase in activated Notch and downstream target genes. We have provided evidence for the first time for increased activation of the Notch pathway in uterine fibroids and a possible role for ADAM12 in this activation.

II. Introduction

Uterine fibroids are benign tumors derived from smooth muscle cells (SMCs) of the myometrium and are the most common tumor of the female reproductive tract. Although most of uterine fibroids are asymptomatic, 20-50% of them will cause clinical symptoms and require treatment [12]. Women with uterine fibroids suffer from heavy menstrual bleeding, pelvic pressure, urinary retention, pain and infertility [5]. Treatment options for fibroids are limited due to their unknown pathogenesis. Currently the mainstay for fibroid treatment is the invasive surgical procedure of hysterectomy, which also removes the ability of patients to bear children [49]. It is essential to find the culprits and pathways involved in uterine fibroid pathogenesis to help discover potential drug targets for therapeutic development.

In an attempt to understand the etiology of fibroids, our lab recently discovered the loss of expression of the tumor suppressor, RE1 silencing transcription factor/neuron-restrictive silencing factor (REST/NRSF), in uterine fibroids [142]. Looking in more detail at the genes effected downstream of REST in fibroids, we found many of its target genes to be aberrantly expressed in the GSE database. One gene in particular, ADAM12 caught our interest due to its role in the development of other tumors and diseases [199-202]. The disintegrin and metalloproteinase-12 (ADAM12) is reported to be involved in extracellular matrix (ECM) remodeling and decidualization [197, 198]. Furthermore, ADAM12 activates downstream pathways by increasing the availability of ligands of growth factor receptors through its catalytic activity [205-207]. Work by Dyczynska and colleagues has shown ADAM12 to cleave Delta-like 1(DLL-1), one of the ligands of the Notch receptor [245]. Notch is a cell-surface receptor involved in cell contact-dependent signaling. The Notch receptor family has five ligands DLL-1, 3, 4 and the Jagged members (JAG-1, 2) [148]. After binding to its ligand, the Notch receptor is

cleaved twice, by a member of the ADAM family and γ-secretase releasing its intracellular domain (NICD) which regulates transcription of target genes [149, 150]. Cleavage of the extracellular domain of liganded Notch by ADAM family member is the rate limiting step for the canonical Notch signaling cascade [246]. Notch has been shown to be important in cell survival and is aberrantly expressed in many diseases [151]. ADAM12 is upregulated in mesenchymal, neural, spleen and liver stem cells which also have high Notch activity [247]. The Notch receptor is reported to be cleaved by ADAM10 and 17, however the possibility of cleavage of the receptor by ADAM12 in pathological diseases has not been investigated [149, 248]. Currently there is a complete lack of information on the role of the Notch pathway in uterine fibroid pathogenesis. In this study, we provide evidence for the first time for a role for ADAM12 in Notch activation in uterine fibroids.

III. Results

1. Nuclear Translocation of Activated Notch is Increased in Fibroids

In an effort to analyze the role of the Notch pathway in uterine fibroid pathogenesis, we initially investigated its activation status in the tumors. When the Notch receptor is cleaved, it releases its intracellular domain, NICD, which travels to the nuclease where it can bind to nuclear effectors and regulate transcription [150]. The full-length Notch receptor and its cleaved form will both be detected by an antibody against Notch, however, they can be individually identified by immunofluorescence (IFC) based on their location in the cytoplasm or the nucleus and by Western blot based on their size. In this experiment, we used IFC to stain for Notch1/NICD1 in matched myometrial and fibroid tissue sections. Our results show an increase in nuclear NICD1 in fibroid sections compared to matched myometrium (Fig. 14).

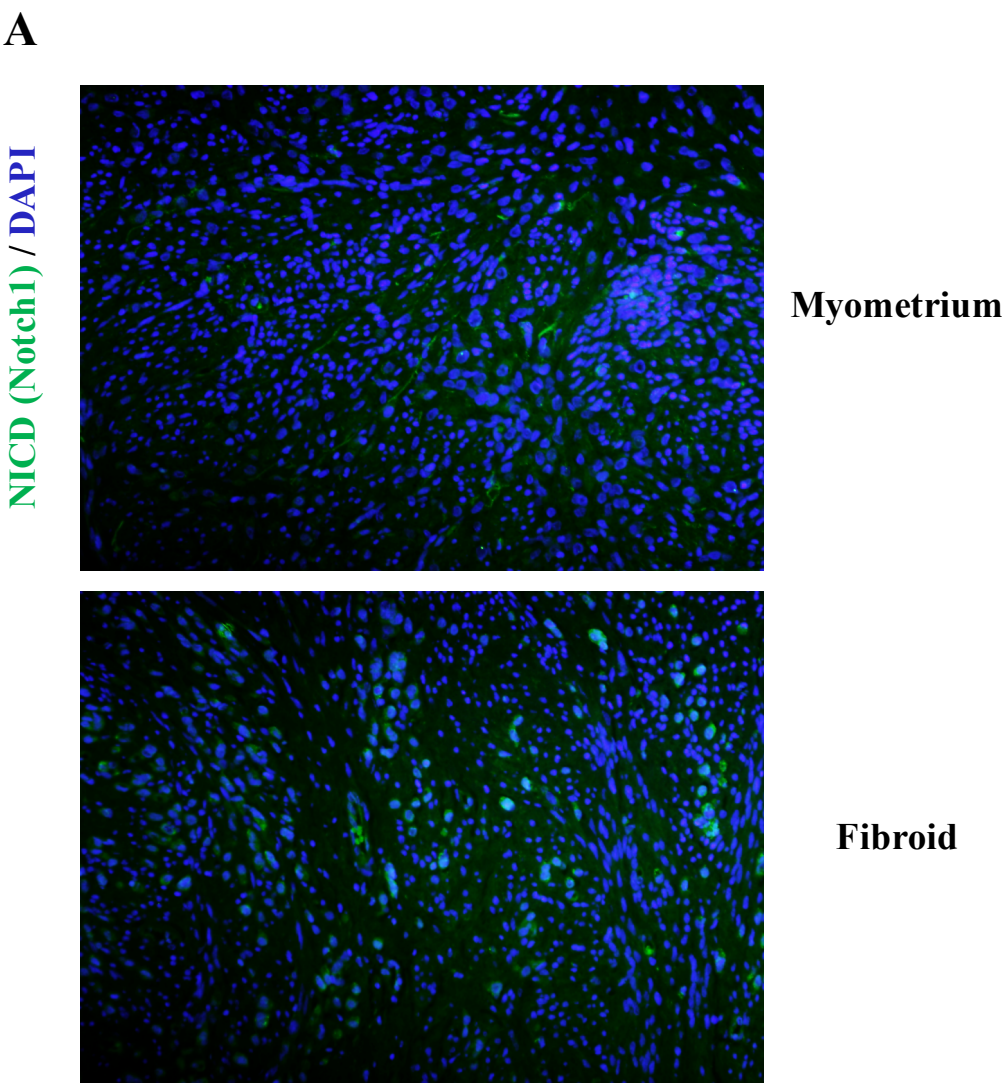

Figure 14: Notch1 expression and NICD nuclear translocation is increased in uterine fibroids. Immunofluorescence staining of myometrial and fibroid tissues with anti-Notch1/NICD (green) antibody and nuclei are stained with DAPI (blue) (n=4).

2. Notch Signaling Pathway Target Genes Are Aberrantly Expressed in Uterine Fibroids

Next, we tested whether the increased activation of Notch1 in uterine fibroids leads to increased transcription of downstream genes. Notch is an oncogenic transcription factor, and when its cleaved form is complexed with the transcription machinery it leads to activation of target genes. [249]. Notch signaling pathway is known to have more than 30 target genes, with roles in apoptosis, cell cycle, differentiation and transcription [250]. In this study, we analyzed the expression levels of Notch target genes, hairy and enhancer of split 1 (Hes1), Hes related family bHLH transcription factor with YRPW motif 1 (Hey1), activating transcription factor 2 (ATF2), and cyclin D1 (CCND1). Using Taqman qRTPCR we found a significant increase in *ATF2* and *CCND1* and non-significant increase of *Hey1* mRNA levels in fibroid samples compared to matched myometrium (Fig. 15A). In addition, *Hes1* mRNA levels were significantly decreased in fibroids (Fig. 15A), while protein expression using IFC showed significant increase of Hes1 in the tumors (Fig. 15B). Our results show for the first time an increase in Notch target genes in uterine fibroids, which suggests an increased activation of the Notch pathway in these tumors. The discovery of new pathways involved in growth and proliferation which may be important in fibroid etiology, provides new possibilities for development of drug therapies.

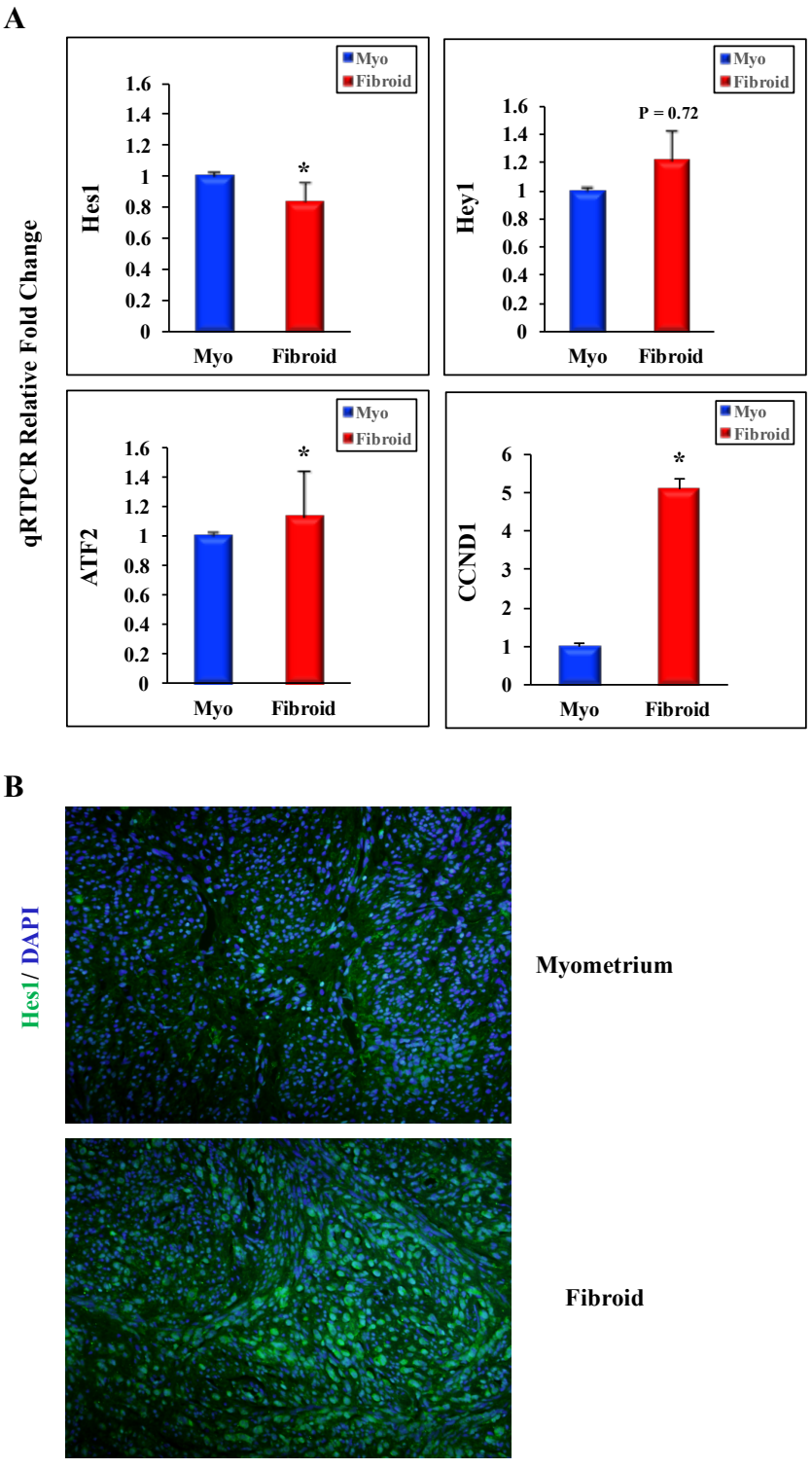

Figure 15: Expression of Notch target genes are increased in uterine fibroids. (A) TaqMan qRT-PCR analysis of Notch target genes *Hes1*, *Hey1*, *ATF2* and *CCND1* expression in 8-15 pairs of matched normal myometrial and fibroid samples. (B) Immunofluorescence staining of myometrial and fibroid tissues with anti-Hes1 (green) antibody and nuclei are stained with DAPI (blue) (n=4). Error bars indicate ± SD, *P < 0.05. Statistical analyses were performed by Mann-Whitney U Test.

3. Notch Pathway Activation is Not Due to Increased Levels of Ligands

In order to understand what is leading to Notch pathway activation in fibroids, we looked at the status of its ligands. In this experiment, we analyzed protein levels of DLL-1 and JAG-1 in myometrial and fibroid matched samples using Western blot. Our results show a significant decrease in JAG-1and a non-significant decrease of DLL-1 protein expression levels in fibroid samples compared to matched myometrium (Fig. 16). These data suggest an increase in JAG-1 and DLL-1 Notch ligands is not responsible for increased Notch1 activation.

4. ADAM12 Overexpression Leads to Activation of Notch Pathway

Previous work has shown the role of ADAMs in cleavage of the Notch receptor [149, 248]. In addition, ADAM12 has been shown to cleave DLL-1 ligand [245]. Therefore, we became interested in further studying the role of ADAM12 in activating the Notch pathway and its target genes in uterine fibroids. For this experiment, we designed an adenoviral vector bearing the ADAM12 cDNA, which we used to over-express ADAM12 in our cells of interest. Here, we transduced primary myometrial SMCs with the adenovirus in 2% FBS media for 48 hours. Analysis of protein from these treated cells showed a significant increase in NICD by Western blot and IFC (Fig. 17B, 17C). Upon further examination of the Notch target genes, we found a significant increase in *Hey1* mRNA (Fig. 17A) and Hes1 mRNA and protein levels (Fig. 17A, 17C) with increased expression of ADAM12 in primary myometrial SMCs. Our data support for the first time a role for ADAM12 in Notch1 activation in uterine fibroids.

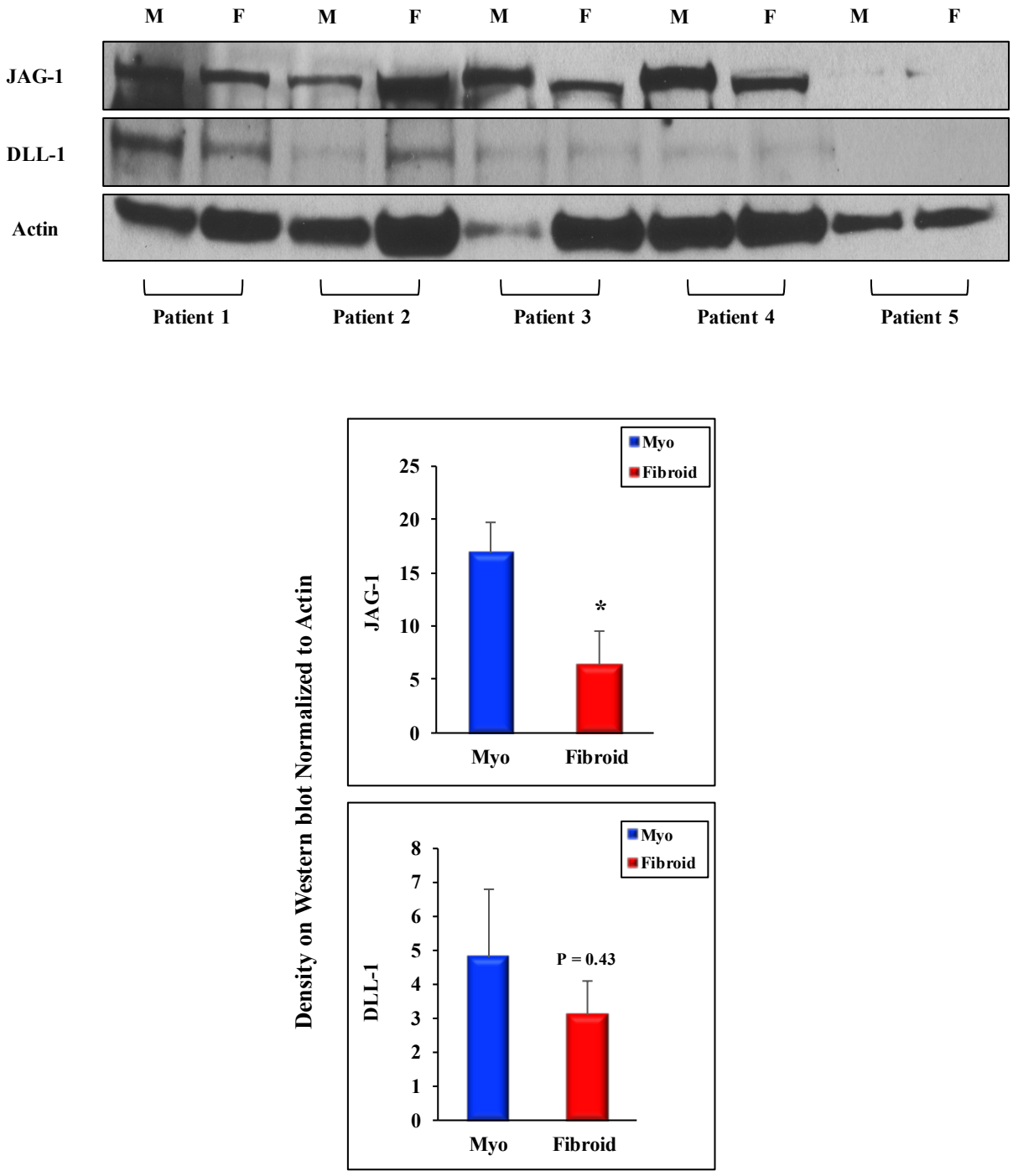

Figure 16: Expression of Notch ligands is decreased in uterine fibroids. Western blot and densitometric analysis of Western blots of protein extracts from patient samples (1-5) comparing JAG-1 and DLL-1 expression in normal myometrium to matched fibroid samples. β-actin was used as protein loading control. Error bars indicate ± SD, *P < 0.05. Statistical analyses were performed by Mann-Whitney U Test.

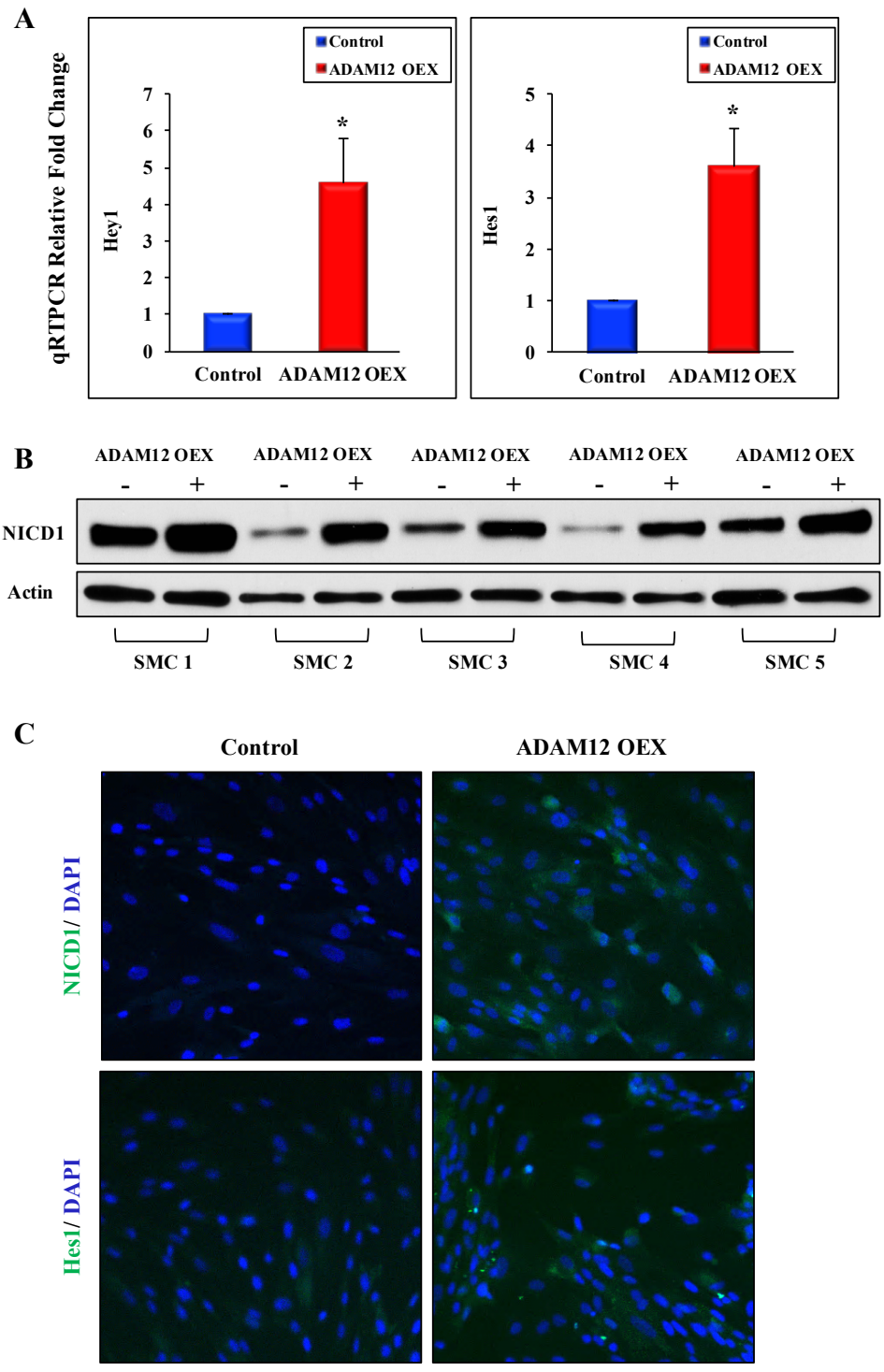

Figure 17: ADAM12 overexpression activates the Notch pathway in myometrial cells. Primary myometrial SMCs treated with adenoviral plasmids containing *ADAM12* cDNA or mock transduced for 48 h were used (A) for RNA extraction and analyzed by TaqMan qRTPCR comparing *Hes1* (n=8) and *Hey1* (n=5) expression levels and (B) protein extraction analyzed by Western blot comparing NICD1 expression levels (n=5) where β-actin was used as protein loading control and (C) immunofluorescence staining of the cells with anti-NICD1 (green) in the top panel and anti-Hes1 (green) antibody in the bottom panel and nuclei are stained with DAPI (blue) (n=4). Error bars indicate ± SD, *P < 0.05. Statistical analyses were performed by Mann-Whitney U Test.

5. REST Regulates Activation of Notch Pathway in Primary Myometrial Cells

Our previous findings in this manuscript suggest a role for REST as a direct and or indirect regulator of ADAM12 and microRNA-29 (miR-29) expression. Furthermore, our data shows a role for miR-29 in regulation of ADAM12 in fibroids. Recent work by Li and colleagues also shows a connection between miR-29 and ADAM12 in NIH3T3 cells. They suggest Notch signaling upregulates expression of ADAM12 through down regulation of miR-29 in these cells [247]. Based on this information and our findings on the effect of ADAM12 overexpression on Notch activation in myometrial cells, we became interested in learning about the role of REST in regulation of Notch in fibroids. To address this question we knocked down REST in primary myometrial SMCs using siREST. We found using Western blot analysis that the loss of REST led to an increase in Notch receptor processing, suggesting an increase in activation of Notch pathway (Fig. 18).

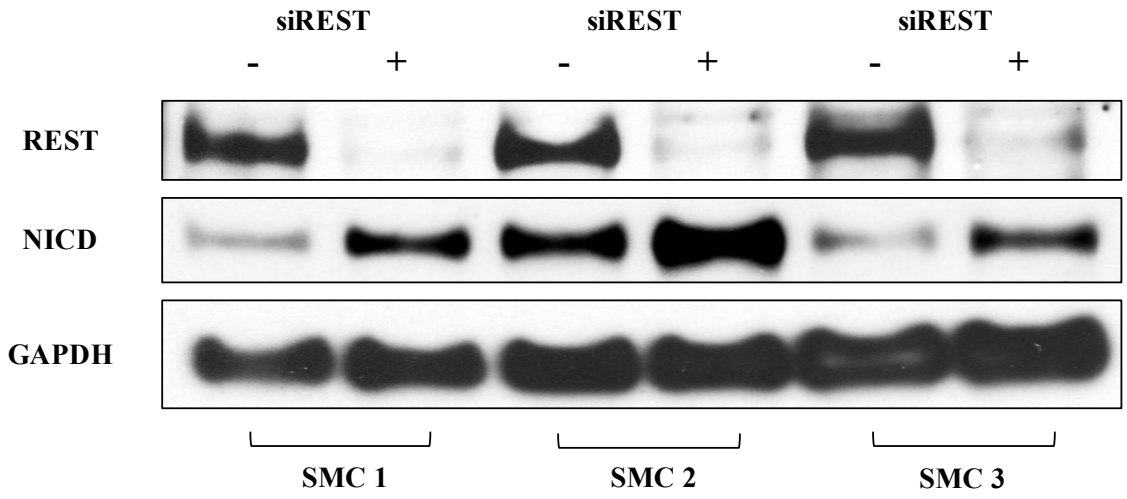

Figure 18: Loss of REST increases Notch receptor processing in myometrial cells. Western blot analysis of protein extracts from myometrial SMCs, from 3 patients, silenced with siREST and mock silenced cells comparing NICD expression levels. GAPDH was used as protein loading control.

IV. Discussion

Uterine fibroids are the most common tumor of the female reproductive tract. However, due to its unknown etiology, there is lack of long term therapeutic options which will leave fertility intact [32]. Understanding the role of signaling pathways in fibroid tumor formation could help in identification of new targets for drug development. In an effort to identify dysregulated pathways in uterine fibroids, we found a lack of knowledge on the role of the tumorigenic Notch pathway in fibroid tumors. Furthermore, previous work has shown ADAM12, an aberrantly expressed enzyme in fibroids, to cleave a ligand of the Notch receptor [245]. The Notch receptor itself is also known to be cleaved by ADAM10 and ADAM17, however, information on possible cleavage by other ADAMs in pathological diseases is lacking [149, 248]. In this study, we investigated the activation status of the Notch pathway and the role of ADAM12 in its activation in uterine fibroids. We provide evidence for the first time for increased activation of the Notch1 receptor and its target genes in fibroid samples and ability of ADAM12 to induce this activation in primary myometrial cells.

Once the Notch receptor is activated by ligand binding followed by two cleavage steps, its intracellular domain, NICD, travels to the nucleus to regulate transcription of target genes [150]. The first proteolytic cleavage of the extracellular domain of liganded Notch by an ADAM family protein is considered to be the rate limiting step in Notch activation. Our data show an increase in nuclear translocation of NICD1 in fibroid tumors along with an increase in target genes *ATF2*, *CCND1* and Hes1. This information suggests an increase in Notch pathway activation in uterine fibroids through Notch receptor 1. In mammals, there are four members of the Notch receptor, Notch 1-4, and more than 30 identified target genes with varying functions [148, 250]. In order

to have a more comprehensive understanding of the Notch pathway in fibroids, further studies are needed to investigate the role of the other Notch receptors and target genes in fibroid tumors.

The Notch receptor ligands include the Delta-like ligands (DLL-1, 3 or 4) and Jagged members (JAG-1, 2) [148]. In order to find if aberrant expression of Notch ligands lead to its activation in fibroids we analyzed the levels of DLL-1 and JAG-1 and found them to be decreased in the tumors. However, the concentration of Notch ligands is not the only parameter to be considered. The activation of Notch signaling depends on its method of interaction with its ligands, where a trans interaction with neighboring ligands activates and cis interaction with a ligand on the same cell inhibits this receptor [251]. Previous studies have shown DLL-1 to be cleaved by ADAM12 in the cis orientation, therefore making available trans positioned ligands for binding and activating the Notch Receptor. In addition, they show processing of DLL-1 by ADAM12 activates Notch signaling in a cell autonomous matter in fibroblast cells [245]. Shortly after, the same research group found ADAM12 shedding of DLL-1 in myoblast cells led to a decrease in Notch activation. The authors propose the proteolytic processing of DLL-1 by ADAM12 leads to a ligand asymmetry which either activates or inhibits Notch signaling in a specific cell and could be important for cell fate determination [252]. In addition, cleavage of the ligand activated Notch by ADAM protease triggers the endocytic recycling of the ligand along with the Notch extracellular domain, within the signal-sending neighboring cell (Fig. 19). Steady state level of Notch ligands may therefore be lower in tissues with activated Notch pathway. In our study, we have found despite the low levels of ligands there is an increased Notch pathway activation, which could be due to proteolytic cleavage of cis ligands supporting Notch activation in uterine fibroids. Additional studies are needed to better understand the specific orientation of the

ligands to the Notch receptors, and also to analyze the role of other DLL and JAG ligands in Notch pathway activation in fibroids.

In order to analyze the effect of aberrantly expressed ADAM12 in fibroids on the Notch pathway, we overexpressed ADAM12 in primary myometrial SMCs and found an increase in Notch activation and downstream target gene levels. These results suggest a role for ADAM12 in Notch pathway activation in fibroids. Work by Li and colleagues indicate a different relationship between Notch and ADAM12, where Notch signaling upregulates expression of ADAM12 through down regulation of microRNA-29 (miR-29) in NIH3T3 cells [247]. Additionally, Diaz et al. found Notch activation in squamous cell carcinoma cell lines to lead to an increase in ADAM12 levels and shedding of heparin-binding EGF-like growth factor (HB-EGF) [253]. It is important to note the experiments by Li et al. were mainly performed in marine derived cell lines [247], and additional work in human cells and in vivo models can help further our understanding of the relationships between Notch, miR-29 and ADAM12. Together, our data providing evidence for loss of REST and ADAM12 overexpression leading to the activation of the Notch pathway and other studies indicating Notch to increase ADAM12 levels, support a possible feedforward loop between ADAM12 and Notch, with REST as a switch (Fig. 20).

In conclusion in this study we have shown for the first time an increase in Notch1 activation and downstream target gene expression in uterine fibroids and a possible role for ADAM12 in this activation. As Notch has important functions in cell proliferation, survival and growth it could be involved in uterine fibroid pathogenesis and potentially be targeted for treatment development. Additional studies on Notch ligands, receptors and target genes are needed to fully understand its role in fibroids.

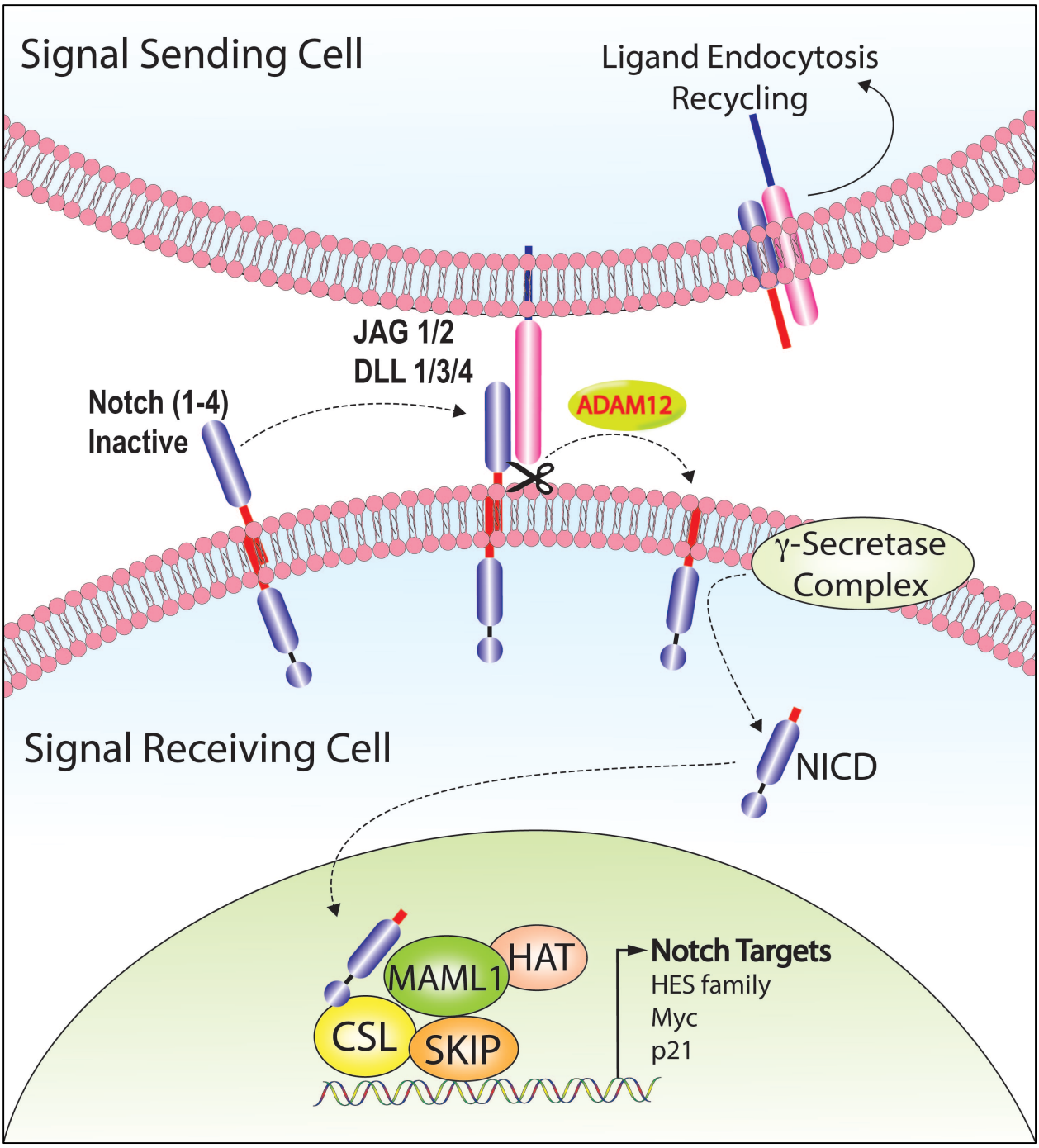

Figure 19: Working model representing cleavage of the ligand bound Notch by ADAM12, triggering the endocytic recycling of the ligand along with the Notch extracellular domain within the signal-sending neighboring cell

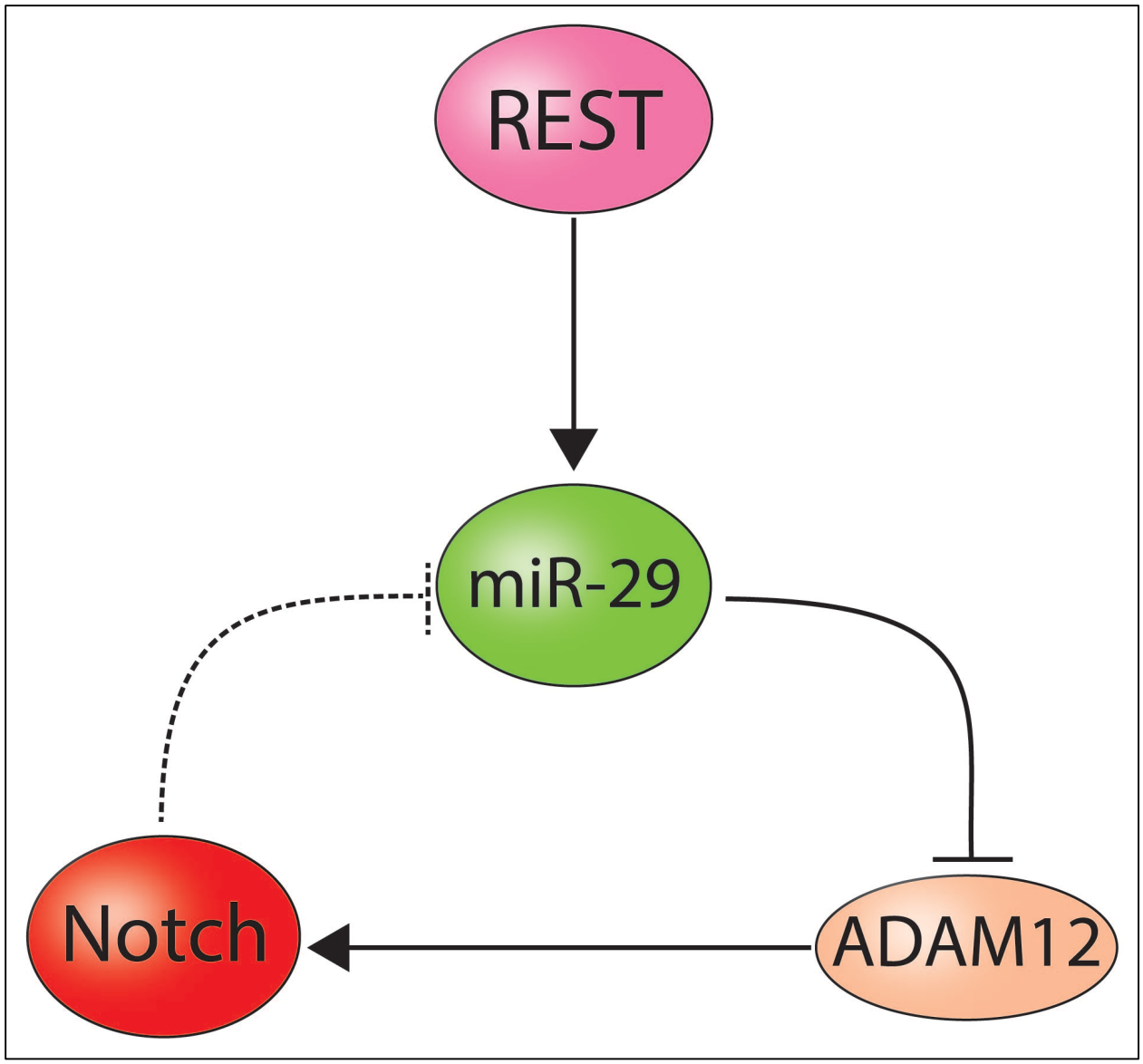

Figure 20: Working model depicting a pathway linking the loss of REST and miR-29 leading to loss of inhibition of ADAM12 and downstream activation of Notch which in turn may inhibit miR-29 expression in the myometrium.

Chapter V: General Discussion

Uterine fibroids are benign tumors of the smooth muscle cells (SMCs) of the myometrium. It is estimated fibroids form in up to 70% of Caucasian and 80% of black premenopausal women [1]. The most common treatment for fibroids is hysterectomy, a surgical procedure which removes the uterus and ability of women to bear children. Hysterectomy is the only definite long term therapeutic option for fibroids and accounts for 76% of all procedures performed for fibroid treatment [49]. In the field of fibroids, there is a lack of an effective, long-term therapeutic option which will also leave fertility intact. This shortage in treatment options is mainly due to the unknown pathophysiology of these tumors [220]. Finding new players and pathways in fibroid etiology is essential for identification of new targets for development of therapeutic options. In this report we have provided evidence for novel druggable players and pathways in uterine fibroids.

Recently, our lab discovered the tumor suppressor REST to be lost in uterine fibroids [142]. Looking in more detail down stream of REST, we found knocking down REST in primary myometrial cells led to an increase of its target genes *NEFH*, *STMN2* and *GRIN2A*. Showing for the first time a connection between loss of REST and aberrant expression of these genes in uterine fibroids. Furthermore, we found ADAM12, a highly expressed metalloproteinase to be a potential target of REST by the Ingenuity® pathway. We provide evidence for the first time for a connection between REST and ADAM12 in uterine fibroids through miR-29. We found silencing REST in primary myometrial cells leads to an increase in ADAM12 and decrease in miR-29 expression levels. Our data suggest a novel role for REST as a positive regulator of miR-29 transcription in fibroids. Additionally, with the use of miR-29 mimics and inhibitors we have found ADAM12 to be negatively regulated by this family of microRNAs. ADAM12 is

upregulated in many cancers and has been associated with growth factor pathway activation through shedding of their ligands [199, 200, 205, 207]. To further understand the role of ADAM12 in pathway activation in fibroids, we overexpressed ADAM12 in primary myometrial cells. Upon this increase in expression of ADAM12 we found an increase in activation and phosphorylation of PI3K-Akt-mTOR, EGFR, p38 MAPK and Notch tumorigenic pathways. In the literature there is a lack of information on the Notch pathway in uterine fibroids. We provide evidence for the activation of the Notch pathway and its target genes *ATF2*, *CCND1* and Hes1in fibroids and a role for ADAM12 in their activation.

ADAM12 inhibition has been an active area of research and several synthetic inhibitors have been developed to inhibit its catalytic activity. KB-R7785 is one such inhibitor, which has been shown by Asakura and colleagues to prevent cardiac hypertrophy by stopping ADAM12 catalytic shedding of HB-EGF [207]. Furthermore, Oh et al. found four compounds targeting the Zn^{2+} binding site to be potent inhibitors of ADAM12 [209]. A more specific inhibitor was designed by Miller et al. targeting the recombinant prodomain of ADAM12. This inhibitor did not cross-react with other ADAM members, and it specifically targeted ADAM12 activity [210]. As an activator of multiple tumorigenic pathways in uterine fibroids, specific inhibition of ADAM12 could simultaneously target the activation of these pathways as well. A multi-target drug could have a more efficient therapeutic effect for uterine fibroid treatment. Research has shown compensatory mechanisms to overcome the benefit of inhibition of a single target, while inhibiting multiple targets together could decrease this compensation [254] When designing therapeutic drugs, it is important to strive for tissue specificity and to minimize side-effects. The use of viral vectors for delivery of inhibitors and direct injection in to tissue of interest could aid in achieving this specificity [113].

Although we provide novel pathways and players in fibroid pathogenesis, our studies were limited to in vitro systems. With the use of primary smooth muscle cells in our experiments we attempted to study conditions as close to the source as possible. Further analysis of our findings and their functions in animal models and in vivo systems is needed to fully understand their role in fibroid pathogenesis. The use of xenograft models will be helpful in testing the effect of inhibitors on growth and proliferation of uterine fibroid tissue.

In conclusion we have reported a novel pathway connecting the loss of REST and decrease in miR-29 leading to loss of inhibition on ADAM12. Furthermore, this increase in ADAM12 leads to aberrant activation of signaling pathways PI3K-Akt-mTOR, EGFR, p38 MAPK and Notch in myometrial cells. Our data have expanded our current knowledge on uterine fibroid etiology and is a step forward towards development of new and effective long term therapeutic options for fibroid treatment.

Materials and Methods

Chemicals and Reagents

Dulbecco's Modified Eagle's medium (DMEM) and bovine calf serum (BCS) were purchased from Sigma Aldrich. FBS, penicillin–streptomycin (pen/strep) and L-glutamine were obtained from Invitrogen. And Dulbecco's PBS was purchased from Thermo Fisher. Gibco® Collagenase type II was obtained from Thermo Fisher. TaqMan primer-probe sets and siRNA reagents were obtained from Thermo Fisher Applied Biosystems and IDT and are listed in *Table* 1. Locked nucleic acid probes and controls were obtained from Exiqon. Antibodies used for Western blotting, immunofluorescence, and ChIP assays and companies obtained from are listed in Table 2.

Protein Isolation and Western blot

Frozen tissue samples were homogenized in lysis buffer (10% Glycerol, 62.5 mM Tris pH 6.8, 2% SDS, 5 mM sodium orthovanadate) supplemented with 1% protease and phosphatase inhibitor mixtures (Sigma-Aldrich). Cultured cells were incubated with lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.5% SDC, 0.1% SDS, 5 mM EDTA) supplemented with 1% protease and phosphatase inhibitor mixtures (Sigma-Aldrich). The homogenates were centrifuged at 12,000 × g at 4 °C. The supernatants were collected to be used in future experiments. For Western blots, aliquots of protein were electrophoresed in 4-15% TGX™ Precast Gels (Bio-Rad), and the proteins were transferred onto nitrocellulose membranes (Thermo Fisher). The membranes were incubated with TBS containing 0.1% TWEEN ® 20 (TBS-T) (Sigma-Aldrich) and 5% nonfat dry milk (NFM) at RT for 2-4 hr and then incubated with a primary antibody and corresponding dilution listed in Table 2 in TBS-T containing 5%

NFM or bovine serum albumin overnight at 4 °C. After washing, the membranes were incubated with the appropriate HRP-conjugated secondary antibody in TBS-T containing 5% NFM at RT for 1 hr. After further extensive washing the membranes were exposed to SuperSignal West Pico Chemiluminescent Substrate, an enhanced chemiluminescent (ECL) HRP substrate (Thermo Fisher) before detection.

Tissue Collection and Cell Culture

Uterine fibroid and patient matched normal myometrial samples were obtained by R.A.N. under institutional review board-approved protocols from the University of Kansas Medical Center from patient approved, premenopausal women undergoing hysterectomy at KUMC hospital. Smooth muscle cells were prepared from the samples by 3-4 hour digestion in 0.5% collagenase II enzyme and were cultured in full fibroid media (DMEM media, 5% FBS, 5% BCS, 1.2% pen/strep,1.6% L-glutamine) to grow into single layered cells.

siRNA Knockdown of REST and MiR-29 Treatment

siRNA knockdown of RE-1 suppressing transcription factor (REST) in primary myometrial SMCs was performed using Silencer Select siRNA (equal mix of s11932, s11933, s11934) from Ambion (Thermo Scientific) with Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific). SMCs were transfected at 80-90% confluency using company protocol and RNA and protein expression were analyzed 48 h after transfection. Control experiments included mock transfections. For miR-29 treatment, cultured primary fibroid cells were transfected with miRCURY LNA™ microRNA mimics (472650-001, 473486-001) and their negative control (479903-001) from Exiqon. For inhibition of miR-29, cultured primary myometrial SMCs were transfected with miRCURY LNA™ microRNA inhibitors (4100754-001, 4100170-001) and

their inhibitor (199006-001) from Exiqon. The cells were transfected under similar conditions as siRNA knockdown of REST.

RNA Collection and qRT-PCR Analyses

Total RNA was isolated from tissue samples or cultured cells using Trizol from Ambion (Thermo Scientific) according to manufacturer's instructions. After quantitation using Nanodrop spectrophotometer, aliquots of RNA were reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Life Technologies, Applied Biosystems). TaqMan assays for primer probes listed in table 2 were used to quantify gene expression using the delta delta C(T) method in comparison with 18s and U6.

Adenoviral Construct Synthesis and ADAM12 Overexpression

The human ADAM12-L plasmid in the pBABE-puro vector was generously gifted to us by Dr. Zolkiewska from Kansas State University (Fig. 21). The ADAM12-L cDNA was amplified using primers (FOR- CAC CTG CAC TGA AGG CCG GCG ACG ATG) and (REV- CAC CTG CAC TGA AGG CCG GCG ACG ATG). The amplified cDNA was then cloned into the pENTR™ /D-TOPO® entry vector and transformed into competent One Shot® TOP10 Chemically Competent *E. coli* cells from Invitrogen (Thermo Scientific) following manufactures instructions. The plasmid DNA was then recombined in to Gateway®-adapted ViraPower™ adenoviral expression vector, pAd/CMV/V5-DEST™ vector from Invitrogen (Thermo Scientific). Adeno-X™ qPCR titration kit from Clontech was used for viral titration according to manufactures instructions. For ADAM12 overexpression experiments, primary cultured myometrial cells at 80-90% confluency were transduced with 30,000 virus particles/cell to achieve a physiological level of ADAM12 similar to their expression in fibroid samples.

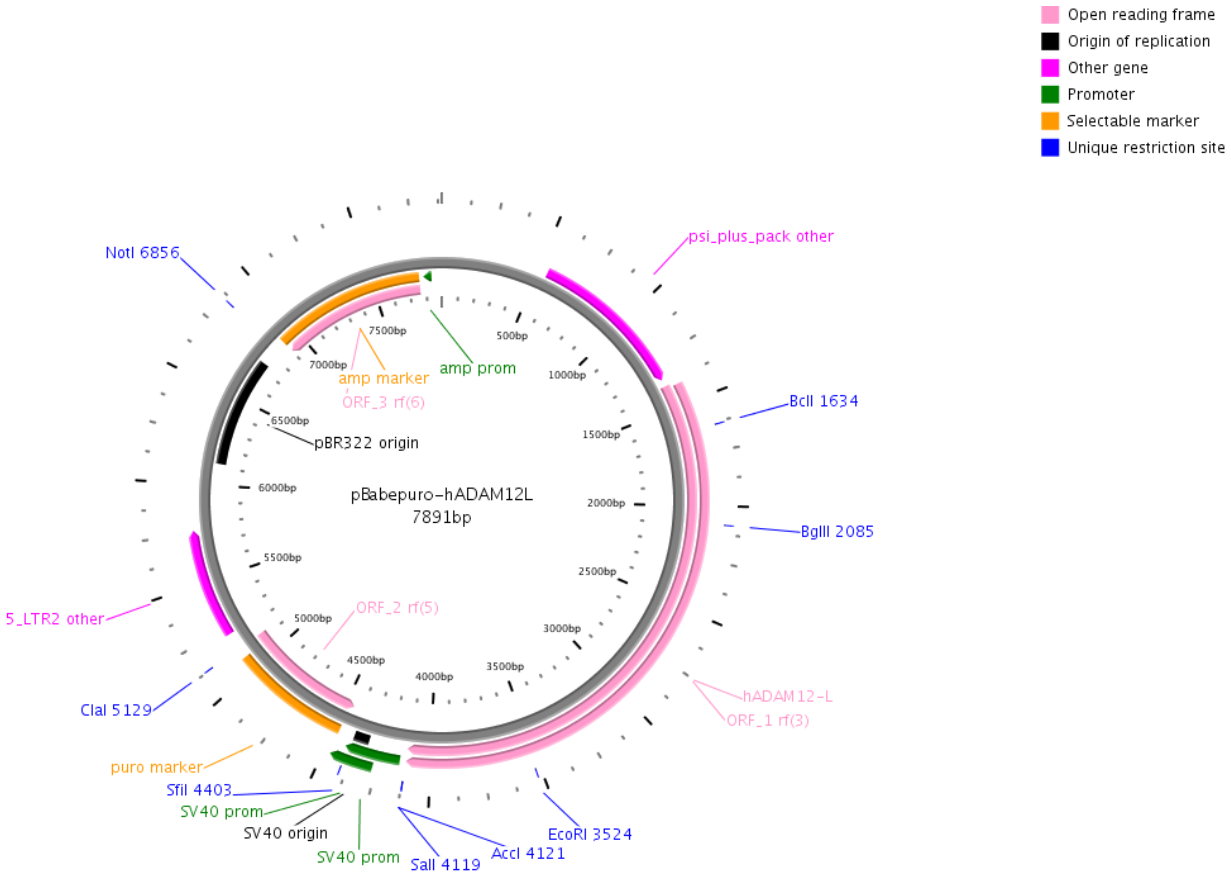

Figure 21: pBabe human ADAM12-L wt map (provided by Zolkiewska laboratory, Kansas State University)

Chromatin Immunoprecipitation

Frozen myometrial and fibroid tissue samples were cross-linked and prepared for chromatin immunoprecipitation (ChIP) by following manufacturer's instructions by SimpleChIP® Enzymatic Chromatin IP (Cell Signaling). The antibodies used for immunoprecipitation included anti-REST and anti-Acetyl-Histone H3 ChIPAb+ antibodies (Millipore), positive control Histone H3 (D2B12) XP® Rabbit mAb #4620 and negative control Normal Rabbit IgG #2729 provided with the SimpleChip kit and anti-RNA polymerase II (Millipore). The DNA eluted from antibody/protein G magnetic beads were analyzed by PCR with primers for REST (FOR- CAG AGA GGG TTT CCT TCA GGT TC, REV- GCT GCA CTT CCC CAT ATC ACT TC), miR-29a (FOR- AGC AGT CAG CAT CAT GGT GCT C, REV- CAG ACT CAT TCC ATT GTG CCT G) and miR-29b (FOR- GAG ACC TGA CTG CCA TTT GTG, REV- TGC GCT GCA CTA CCA ACA G)

Immunofluorescence

Uterine tissues were fixed in 4% paraformaldehyde and processed for paraffin embedding. Tissue sections (5-μm thickness) were deparaffinized in xylene, rehydrated through a series of ethanol washes. Rehydrated tissue sections were subjected to antigen retrieval by heating in citrate buffer (Vector Laboratories). When cells cultured on chamber slides (ibidi) were used for ADAM12 overexpression studies, they were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 before immunofluorescence experiments. The slides were washed with PBS, blocked for 30 min in blocking agent 5% NFM followed by primary antibody in blocking buffer overnight at 4 °C. The slides were further washed in PBS and incubated with Alexa Fluor 488 labeled secondary antibody (Invitrogen, Life Technologies) for 1 h at 37 °C. The slides were

then mounted with ProLong® Gold Antifade Mountant with DAPI for nuclear stain from Life Technologies and were visualized under fluorescence microscope (OlympusIX71).

Table 1: List of primer probes used for qRTPCR.

Gene Name	Sequence Name	Company
NEFH	Hs.PT.58.27071610	IDT
GRIN2A	Hs.PT.58.26949410	IDT
STMN2	Hs.PT.58.5075784	IDT
GRIA2	Hs.PT.58.39408246	IDT
HBEGF	Hs.PT.58.20429993	IDT
SCG2	Hs.PT.58.22473023	IDT
DCX	Hs.PT.58.1252315	IDT
SALL1	Hs.PT.58.22909622	IDT
CBLN1	Hs.PT.56a.38490877	IDT
ATF2	Hs.PT.58.20697093	IDT
HEY1	Hs.PT.58.15477803	IDT
DPT	Hs.PT.58.22664754	IDT
HES1	Hs.PT.58.4181121	IDT
RNA18S5	Hs.PT.39a.22214856.g	IDT
CCND1	Hs.PT.56a.4930170	IDT
COL3A1	Hs00164103_m1	Invitrogen
COL1A2	Hs01028970_m1	Invitrogen
ADAM12	Hs01106104_m1	Invitrogen
has-miR-29a	Assay ID: 002112	Invitrogen
has-miR-29b	Assay ID: 000413	Invitrogen
U6 snRNA	Assay ID: 001973	Invitrogen
ADAM12-S FOR	CTT TCT GCA AAC CCT CAA ACC	IDT
ADAM12-S REV	ACT GGA GAA GAA GGT AGG AGA A	IDT
ADAM12-L FOR	GCT CAG CTC CAA GCA GTA TAG	IDT
ADAM12-L REV	CAC TGC CAC CAG TAG GTT ATT	IDT

Table 2: List of antibodies used for Western blot and immunofluorescence experiments.

Name	Company	Product #	Dilution (WB)	Dilution (IFC)
DCX	Cell Signaling	4604S	1 in 1000	-
GRIA2	Cell Signaling	13607S	1 in 1000	-
REST	Millipore	07-579	1 in 2000	-
ADAM12	Abcam	39155	2 in 3000	-
ADAM12	Zolkiewska laboratory	3394	1 in 10,000	-
EGFR	Cell Signaling	4267S	1 in 1000	-
Phospho-EGFR (Y1068)	Cell Signaling	3777S	1 in 1000	-
Phospho-EGFR (Y1173)	Cell Signaling	4407S	1 in 1000	-
Akt	Cell Signaling	9272S	1 in 1000	-
Phospho-Akt (Ser473)	Cell Signaling	4060S	1 in 1000	-
mTOR	Cell Signaling	2983S	1 in 1000	-
Phospho-mTOR (Ser2448)	Cell Signaling	2971S	1 in 1000	-
p38 MAPK	Cell Signaling	D13E1	1 in 1000	-
Phospho-p38 MAPK (Thr180/Tyr182)	Cell Signaling	D3F9	1 in 1000	-
Notch1 (NICD1)	Cell Signaling	D1E11	1 in 500	1 in 100
DLL1	Cell Signaling	2588	1 in 500	-
Jagged1	Cell Signaling	28H8	1 in 500	-
Hes1	Cell Signaling	D6P2U	-	1 in 100
βactin	Santa Cruz	Sc-1616	1.5 in 2000	-
GAPDH	Cell Signaling	5174S	1 in 3000	-

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